THE DETERMINATION OF MINIMUM VITAMIN REQUIREMENTS FOR GROWTH[1]

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THREE FIGURES

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The minimum requirement for growth of any nutrient may be defined as the lowest level of that nutrient which allows the maximum growth inherently possible. Such a definition tacitly assumes that maximum growth is optimum growth. This is obviously true if one is concerned with meat production, provided the growth is obtained economically, but is much less obviously so when intelligence, longevity, milk production, etc., are the prime considerations. However, the definition appears to be generally accepted as applying to human beings, since recommended allowances have always been set at levels thought to be considerably above minimum requirements. It is also generally accepted that *minimum requirements should adequately cover the needs of all individuals.*[2] If this view is adopted, then the average requirement loses importance if there is a large variation in the data from which it was derived. That is, if there is a considerable

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[2] This statement should perhaps read "99% or 99.9% of all individuals." If the vitamin requirements of individuals fit a normal distribution, those of a few would be infinitely high.

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range in individual needs, a diet which supplies only enough to meet average needs will be too low for a considerable portion of the population. It will also, of course, contain more than enough for a similarly large fraction, but this would appear to be of relatively little importance unless the cost of the nutrient must be carefully considered along with availability.

It would thus appear that the chief danger in estimating requirements is that they will be underestimated since little attention has been paid to differences in the needs of individuals within the population studied. If the range of requirements could be estimated, the recommended allowance could be made with some assurance rather than being arrived at simply by doubling or tripling the minimum needs. Although this has not been accomplished, it does appear to the author that the usual methods of evaluating the data from experiments designed to determine minimum requirements for growth tend to underestimate the needs, and that less critical methods may be justified. In this paper, data collected on the riboflavin requirements of ducklings are used as examples for discussion since they are more or less typical of material which must be evaluated.

EXPERIMENTAL

The experimental details are presented in the paper by Hegsted and Perry ('48). Pekin ducklings 3 or 4 days old were divided into groups of 4 or 5 ducks each and fed purified rations containing different levels of riboflavin ranging from 100 to 500 μg per 100 gm of diet.[3] After 2 weeks the gain per day during the last 10 days on experiment was calculated. The effect of body stores on growth should have been minimized by using only the latter part of the study in the calculation.

[3] The crystalline vitamins other than folic acid used in these studies were kindly supplied by Merck and Company, Rahway, New Jersey; the glucose and corn oil by the Corn Industries Research Foundation, New York, New York; and the folic acid by the Lederle Laboratories, Pearl River, New York.

The problem in the analysis of the data is to determine at what level of riboflavin fed maximum growth is reached. It is also of interest to determine, if possible, whether the response to riboflavin is linear over the entire range of riboflavin supplementation to the point where maximum growth is obtained. While it is unlikely that this latter proposition is true, if it were approximately true the analysis would be greatly simplified. It seemed likely that the response might be more nearly linear when the logarithm of the dose was used rather than the dose itself. Various methods of examining the data which have previously been used were therefore compared, namely (a) plotting the mean gains of the various groups against the riboflavin content, (b) plotting individual gains against riboflavin content, and (c) plotting individual gains against the log of the riboflavin content. In the latter 2 instances the best regression lines were calculated by the method of least squares through the values for the first 3 groups (100 to 300 μg riboflavin/100 gm diet), the first 4 groups (100 to 400 μg riboflavin), and the 5 groups (100 to 500 μg riboflavin).

RESULTS

Mean gain vs. riboflavin level

In figure 1 the mean gain of each of the groups is plotted against the riboflavin content of the diet and a smooth curve drawn through the points by inspection. This is the usual treatment of such data and casual inspection suggests a requirement of about 350 μg of riboflavin per 100 gm of diet. Application of the "t" test does not reveal any significant difference in the gain at the 300 and 400 μg levels of riboflavin in any of the experiments or in the combined data.[4]

Individual gain vs. riboflavin level

When the individual gains are plotted in a similar manner (fig. 2), the requirement for maximum growth is far from

[4] All of the data have been tested at 0.05 probability level for significance. Since the paper is a general discussion of the problems involved, the statistical tables have not been presented.

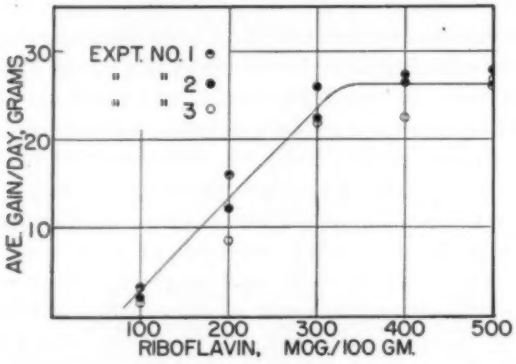

Fig. 1 The average gain per day of the groups of ducklings receiving various levels of riboflavin.

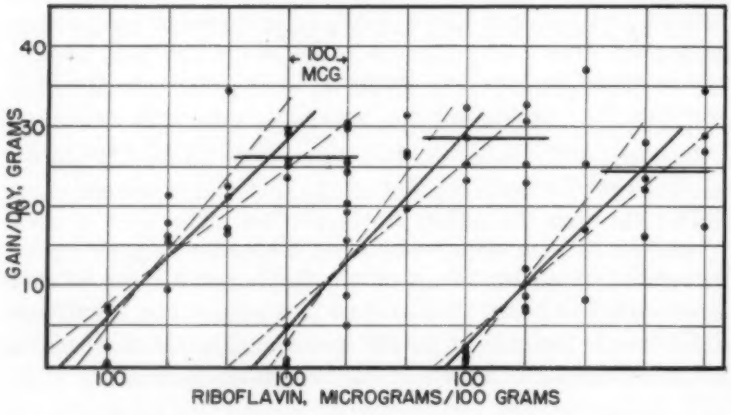

Fig. 2 Scatter diagram showing the gain per day of each duckling versus the riboflavin content of the diet. The solid line is the regression line through the points for the first 4 levels of riboflavin. The solid horizontal bar is drawn through the mean weight of the last 2 groups. The dotted lines are the regression lines for the first 3 groups and all 5 groups.

clear. As judged by the correlation coefficient (table 1) the correlation between gain and riboflavin level is as good over the total range tested, from 100 to 500 μg riboflavin, as it is for the first 3 levels from 100 to 300 μg. The various groups of data were also examined for departure from linearity by the covariance method described by Fisher ('46). As might be expected, none of the data depart significantly from linearity

TABLE 1

Riboflavin requirements as estimated from different regression lines and 2 methods of plotting the data.

EXPERIMENT NUMBER	RANGE OF RIBOFLAVIN IN REGRESSION LINE IN μG/100 GM	UNITS ON ABSCISSA					
		Riboflavin μg/100 gm			Log riboflavin μg/100 gm		
		r [1]	Riboflavin requirement		r [1]	Riboflavin requirement	
			Growth [2]	Maintenance [3]		Growth [2]	Maintenance [3]
1	100–300	0.837	327	47.6	0.858	372	81.3
	100–400	0.868	373	24.0	0.906	380	81.2
	100–500	0.826	434	—38.0	0.858	444	67.3
2	100–300	0.826	341	74.6	0.889	377	95.1
	100–400	0.845	390	48.6	0.901	405	92.5
	100–500	0.824	448	20.0	0.902	450	87.0
3	100–300	0.789	338	94.3	0.759	403	98.6
	100–400	0.797	396	69.7	0.804	434	96.4
	100–500	0.810	431	46.7	0.829	437	96.0

[1] Correlation coefficient.

[2] Riboflavin content at intersection of the regression line with the horizontal line through the mean gain at 400 and 500 μg per 100 gm.

[3] Riboflavin content at intersection of the regression line with the horizontal line through zero gain.

although there is a tendency for greater departure as the range of riboflavin is increased.

If one assumes that the gain over any of the riboflavin ranges tested is linear with respect to riboflavin concentration, then the requirement for maximum growth would be that value of riboflavin at which the regression line reaches a value taken as maximum gain. In these studies we have taken the mean gain of the last 2 groups as maximum gain.

In figure 2 a solid line is drawn horizontally through this value, and the riboflavin value at which the regression line meets this line may be read off of the graph. These values are shown in table 1 as the growth requirement determined by each regression line.

Individual gain vs. log riboflavin level

In figure 3 the data have been plotted as in figure 2 except that the log of the riboflavin content of the diet has been used as the unit of the abscissa. The 3 regression lines

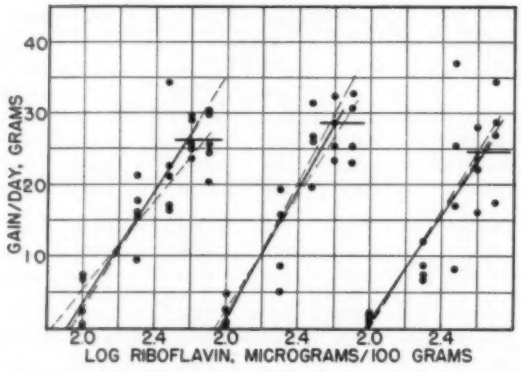

Fig. 3 Scatter diagram showing the gain per day for each duckling versus the logarithm of the riboflavin content. See the legend of figure 2 for explanation of the regression lines.

for each experiment were calculated as before and the requirement for maximum growth read off again as the riboflavin value at which the regression line reaches maximum gain. These values are shown in table 1. It may be seen that the requirement thus determined is less influenced by the choice of the data through which the regression line is calculated than is the case in figure 2. Also it may be noted that in general the use of log riboflavin has increased the correlation coefficient but not to a significant degree.

Maintenance requirement

If the point at which the regression line meets a horizontal line through maximum gain be taken as the requirement for growth, then in an analogous manner the riboflavin value giving zero gain should be the maintenance requirement. These values have also been reported in table 1 as determined in figures 2 and 3 for the various regression lines. It may be seen that this value, whatever its significance, would appear to be fairly reliable when calculated from a log dose-response curve, but of little value as determined from the dose-response curve. If it should be shown that the maintenance requirement is the same for animals of different weights and ages, this value might be useful as a means of determining adult requirements.

TABLE 2

Statistics calculated from the combined data.

STATISTIC	MICROGRAMS OF RIBOFLAVIN PER 100 GM DIET				
	100	200	300	400	500
Mean gain, gm	2.5	12.6	23.4	25.6	26.9
Standard deviation	2.46	5.35	8.02	2.43	4.91
Standard error of mean	0.71	1.54	2.32	0.70	1.42
Coefficient of variation	98.4	42.5	34.4	9.5	18.2

Variability within groups

The second premise made in the introduction was that "minimum requirements should be set sufficiently high to cover the needs of all individuals."[5] It is instructive here to examine the extreme limit of the gains in each group. In each experiment the group at the 300 μg level of riboflavin has a lower limit than the groups at 400 and 500 μg and the range of values is greater. This is demonstrated more clearly in table 2 in which several statistics have been calculated for the combined data from the 3 experiments. Of chief interest

[5] See footnote 2, p. 399.

is the coefficient of variation which tends to decrease with increasing levels of riboflavin. This suggests that at low levels of riboflavin supply one is dealing with more than 1 variable. These may be (a) inherent ability to gain and (b) response to riboflavin.[6] When sufficient riboflavin is supplied to meet the needs of all of the individuals, the second variable is largely removed and the groups become less heterogeneous.[7] It is not unlikely therefore that the 300 μg level is adequate for a considerable proportion of the individuals, but those for which it is inadequate deserve considerable attention.

DISCUSSION

The determination of growth requirements is inherently more difficult than the usual vitamin assay and subject to much greater error. In vitamin assays the work can be done in that range of vitamin supplementation where the response is essentially linear to the dose or the log dose, and by choosing standards and unknowns in such amounts that an equal response is obtained, errors due to inaccuracies in the slope of the regression line may be largely eliminated. In studying requirements, however, one is interested in the extreme portions of the dose-response curve where response may not be linear with respect to dosage, and where errors in the slope of the regression line produce great errors in the value to be determined.

Previous work covering the assay of many vitamins has shown that the log dose-response curve is more nearly linear than the dose-response curve (Irwin, '37; Coward, '38). For the data considered in this paper, this appears to be highly probable although it is not possible to prove statistically. It would appear that the more general use of log dose-response

[6] It may be equally valid to explain the variation in terms of differences in food intake, and thus differences in riboflavin intake.

[7] It may be noted that the variance of the groups is not the same. According to Fischer ('46) this does not invalidate the common "t" test since one is interested only in knowing the probability that the 2 groups are from the same population. A significant value for "t" may be obtained either when the means are different or when the variances are different.

curves would have the following advantages in the determination of requirements: (a) The unreliability of the value obtained is emphasized. For example, on a log scale the difference between 300 and 400 μg of riboflavin is relatively small while on an ordinary scale this represents a large portion of the range being considered. (b) The value obtained for the growth requirement is less dependent upon the judgment of the investigator in selecting the region over which it is valid to calculate the regression line. (c) The maintenance requirement thus determined appears to be a relatively reliable figure.

On the other hand, the usual test is to compare statistically the gains at 2 levels of vitamin supplementation to determine whether 1 level gives a significantly better response, such as the 300 and 400 μg riboflavin level in the present data. This is at best a highly uneconomical procedure for, in effect, one discards the major portion of his data in making the test, and uses the data from only 2 small groups at exactly the point where large numbers are required to prove small differences significant. The statistician will correctly suggest that the experiment should be repeated with a new design so that large numbers fall within these groups. This, however, proves to be highly impractical in most instances. If one combines all of the data available at the 300 and 400 μg levels of riboflavin, one may calculate that at least 70 birds would be needed to show the mean gains to be significantly different. Following this, even larger numbers would be required to test the differences between the 400 and 500 μg levels. Few laboratories could undertake such a problem.

In comparing 2 groups of data, one is ordinarily testing the hypothesis that the 2 groups are the same, i.e., from the same population. Failure to find the differences to be significant may mean that the groups are the same or that the data are insufficient to prove the significance of the difference. It does not prove that the groups are the same. Therefore one must consider the likelihood of making a false decision, and in the small groups usually available in nutrition work,

it is apparent that a false decision will often be reached. One is more likely to conclude that 2 groups are not different when they actually are, than the reverse. Since this is also the more serious error, if one wishes to have requirements high enough to cover the whole population, then he must not rely completely upon the statistical test (see Eisenhart and Wilson, '43, for a clear example of this point).

While every effort should be made to increase the extent of the data and to apply appropriate statistical examination, the judgment of the investigator must not be completely discarded. The present data on the riboflavin requirement of the duckling are inadequate to prove statistically that more than 300 μg of riboflavin per 100 gm of ration are necessary, but other considerations lead us to conclude that probably the requirement is not less than 400 μg and that 500 μg per 100 gm may be a safer level.

CONCLUSION

The usual method used in the determination of growth requirements is to feed various levels of the nutrient and then to test statistically the difference in response between the various levels to determine at which level one ceases to obtain a significant increase in response. Almost invariably small groups of animals must be used. Thus, most of the data are discarded in making the critical test, at exactly the point at which large groups are required to show the small differences to be significant, and the most likely error is that one will conclude that the groups are not different when in fact they may be. This type of error will invariably lead to a low estimate of requirement. A low estimate is considered a more serious error than the less likely over-estimation since one is interested in setting requirements high enough to cover adequately all of the individuals.

It is suggested that by plotting a log dose-response curve and determining the point at which the regression line, drawn through the region of approximately linear response, reaches maximum growth one arrives at a fairer estimate of the true

requirement. The maintenance requirement, that amount of nutrient required to give just zero gain, may also be determined with considerable accuracy from the same regression line.

ACKNOWLEDGMENTS

The author is indebted to Mr. Richard Perry for assistance in the care of the animals and to Miss Virginia Kent for some of the statistical calculations.

He also wishes to thank Drs. Hugo Muench and Jane Worcester, of the Department of Biostatistics, for stimulating discussions and numerous suggestions. However, the author accepts full responsibility for the conclusions of this paper.

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NUTRITIONAL STUDIES WITH THE DUCK

V. RIBOFLAVIN AND PANTOTHENIC ACID REQUIREMENTS [1]

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ONE FIGURE

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In previous publications in this series we have reported studies on the pyridoxine (Hegsted and Rao, '45) and niacin (Hegsted, '46) requirements of ducklings. The present paper presents the results of investigations on the riboflavin and pantothenic acid requirements of this species.

EXPERIMENTAL

The rations [2] used in this study were of the purified type and had the compositions shown in table 1. Either riboflavin or calcium pantothenate was omitted to produce the appropriate vitamin low diet. Day-old Pekin ducklings were received from the hatchery and fed a commercial diet for 3 or 4 days. They were then divided into groups of 4 or 5 ducklings each and given the experimental diets to which had been added various levels of the vitamin under study. Each bird was weighed every other day. The experiments were terminated after 2 weeks and the gain per day during the last 10 days was

[1] Supported by grants-in-aid from the John and Mary R. Markle Foundation, New York; Swift and Company, Inc., Chicago; Milbank Memorial Fund, New York; and the Nutrition Foundation, Inc., New York.

[2] Folic acid was supplied by the Lederle Laboratories, Pearl River; and the other crystalline vitamins by Merck and Co., Rahway. Glucose and corn oil were supplied by the Corn Industries Research Foundations, New York.

calculated for each bird. By using only the data obtained during these last 10 days of the experiment, the influence of body stores on growth should have been minimized.

RESULTS

Symptoms of deficiency

Neither riboflavin nor pantothenic acid deficiency appears to produce characteristic gross symptoms in the duckling. On diets lacking either of these vitamins the birds fail to grow after the second or third day, and usually die within 4 to 7 days. There appears to be an excess of secretion from the

TABLE 1

Composition of the basal ration.

BASAL MIXTURE	%	VITAMIN SUPPLEMENTS TO BASAL MIXTURE	*mg/100 gm*
Glucose	50.7	Thiamine chloride	400
Casein (vitamin-free)	18.0	Pyridoxine hydrochloride	400
Gelatin	10.0	Riboflavin	800
Corn oil	10.0	Calcium pantothenate	2500
Salt mixture (Hegsted et al., '41)	0.5	Nicotinic acid	4000
$CaHPO_4 \cdot 2\,H_2O$	1.0	Biotin	20
Cod liver oil	2.0	Folic acid	100
Cellu flour	3.0	Menadione	100
Choline chloride	0.3	Alpha tocopherol	50,000

eyes, and the eyelids may become stuck together. No evidence of curled toe paralysis or dermatitis, the characteristic symptoms of these deficiencies in the chick, has been noted.

The failure to show symptoms is probably not the result of the severe lack of vitamin in these experiments. The few birds which survive longer periods, and some which have been maintained on suboptimum levels of either of the vitamins, also fail to show characteristic signs. It may be noted that of all the vitamin deficiencies so far studied in ducklings, the only characteristic symptoms which have been observed are the polyneuritis of thiamine deficiency, paralysis in vitamin A

deficiency, and perosis in choline deficiency. Deficiencies of vitamins D, E, and K have as yet not been studied, and pyridoxine deficiency is the only deficiency disease which has been studied in chronic form. The occasional convulsion observed in pyridoxine deficiency is too rare to be considered typical of the disease.

Riboflavin requirements

The data, collected in 3 experiments in each of which 5 levels of riboflavin were fed, were discussed extensively in a previous publication (Hegsted, '48) and need not be presented here. That publication also describes the methods used in evaluating the data. The values for the gain of each animal were plotted against the logarithm of the vitamin concentration in the diet. The regression line was then calculated through all of those groups in which the response appears to be approximately linear to the logarithm of the vitamin concentration. The minimum requirement for growth is taken as the point at which the regression line reaches the mean gain of the groups at the highest levels of intake. A minimum requirement for maintenance may also be calculated. This is the vitamin concentration which gives zero gain according to the regression line.

The results obtained for 3 experiments have been previously summarized (Hegsted, '48). It was concluded that the minimum requirement for growth is approximately 400 μg of riboflavin per 100 gm of diet and that approximately 90 μg per 100 gm is required for maintenance.

Pantothenic acid requirements

Data on the pantothenic acid requirements were obtained in a similar manner in 2 experiments. Figure 1 shows the levels of pantothenic acid fed and the gain per day of each bird. The regression lines were calculated for all of the values from 200 to 1500 μg, inclusive, of calcium pantothenate per 100 gm of ration. The mean requirement for maximum

gain was taken as the calcium pantothenate value required by the regression line to give a gain equal to the average of the 2 groups receiving the highest levels, and the maintenance requirement the amount to give zero gain. The values thus calculated are shown in table 2.

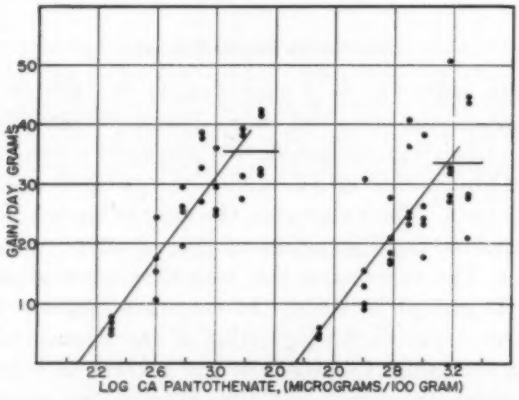

Fig. 1 Scatter diagram showing the gain per day of ducklings which received diets with varying amounts of pantothenic acid. Regression lines were calculated as described in the text.

TABLE 2

Pantothenic acid requirements of ducklings.

EXPT.	NUMBER OF BIRDS USED	AVERAGE MAXIMUM GAIN	CALCIUM PANTOTHENATE REQUIREMENT	
			Growth	Maintenance
		gm/day	*μg/100 gm*	*μg/100 gm*
1	28	35.4	1350	115
2	33	33.5	1415	133
		Mean	1382	124
		As pantothenic acid	1175	105

DISCUSSION

It has been pointed out in a previous discussion (Hegsted, '48) that the method of treating the data may give higher

estimates of requirements for growth than the statistical comparison of the differences of the mean gains obtained for the various levels of vitamin concentration in the diets. This must be borne in mind in comparing these estimates with values in the literature since differences may be of interpretation rather than in the data themselves. However, only 1 paper on the riboflavin requirements of ducklings is available and none on pantothenic acid. Fritz, Archer and Barker ('39) estimated the minimum requirement to be 300 μg of riboflavin per 100 gm of diet. This is in the same general range as previous estimates for chicks which have ranged from 200 to 350 μg per 100 gm of diet (see review by Sherwood and Couch, '45). Lepkovsky and Jukes ('35) concluded that the chick requirement was about 230 μg and that of poults was reported to be approximately the same (Jukes, '38).

The pantothenic acid requirements presented for chicks have varied even more than the estimates of riboflavin requirement. Jukes ('39) estimated it to be 1400 μg per 100 gm of diet while Bauernfeind et al. ('42) concluded that 600 μg per 100 gm was more nearly correct. Recent studies by Lepkovsky et al. ('45) suggest values between 460 and 970 μg per 100 gm of diet for chicks and between 970 and 1170 for turkey poults. If the requirements of ducklings are similar to those of these species, our figures would favor the higher estimates.

Lepkovsky et al. ('45) suggest that requirements may vary with the rate of growth. While more carefully controlled studies are clearly needed to demonstrate this, it would appear to be highly probable. In this respect it is disconcerting to note that the maximum gain attained on the riboflavin studies was considerably below that observed in the studies on pantothenic acid. No explanation is available other than the possibility that this is due to a seasonal variation perhaps affecting the diets of the laying birds. It is of course possible that the riboflavin requirements would have been higher had maximum growth been obtained, but this seems unlikely in view of the fact that the estimated requirements are already above those reported for other species.

Although the practical significance of the maintenance requirement calculated in these studies is problematical, it is of interest to note that approximately 10 times the maintenance value of pantothenic acid was needed for maximum growth, whereas with riboflavin only 4 to 5 times the maintenance value was needed.

It is realized that requirements should probably be related to the composition of the diet. Fat (Mannering et al., '44) has been observed to increase riboflavin needs and high levels of protein appear to lower pantothenic acid requirements (Nelson et al., '47). However, the best estimates of requirements which can be made may be subject to errors great enough to obscure these effects under practical conditions.

CONCLUSION

Studies on the riboflavin and pantothenic acid requirements for growth of ducklings have yielded estimates of approximately 400 μg of riboflavin and 1100 μg of pantothenic acid per 100 gm of diet. Neither of these deficiencies produces characteristic symptoms in the duckling.

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FALSE HIGH VALUES FOR ASCORBIC ACID IN GUAVA JUICE

A NOTE ON THE USE OF THE COLORIMETRIC METHOD WITH 2,4-DINITROPHENYLHYDRAZINE[1]

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In the course of some studies on the stability of ascorbic acid in bottled guava juice the colorimetric method of Roe and Oesterling ('44) for total ascorbic acid was applied. The values obtained were much higher than those found with the usual dye-titration method. Samples which by the titration method showed complete loss of ascorbic acid gave values indicating practically no loss by the colorimetric method. In an effort to clarify this situation the following studies were carried out.

A number of additional samples of guava juice were assayed for reduced ascorbic acid by the dye-titration method and for total ascorbic acid by the colorimetric method during various periods of storage. The samples were assayed on the day the bottles were originally opened, then recapped and kept in the refrigerator during the experimental storage periods. The results are shown in table 1.

In every case the value obtained by the colorimetric method was as high as or higher than that obtained by dye-titration.

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Also the values obtained by the colorimetric method indicated little or no loss of ascorbic acid during the storage period. On the other hand, the values obtained by dye-titration indicated a rapid and considerable loss of the vitamin.

This discrepancy between the vitamin values obtained by the 2 methods might be accounted for on the basis of the presence of dehydroascorbic acid since only reduced ascorbic acid was determined by the dye-titration method. In order

TABLE 1

Comparison of values for ascorbic acid in guava juice as determined by the dye-titration and colorimetric methods. All values are in mg/100 ml of juice.

SAMPLE	WEEKS IN ICE BOX	METHOD	
		Dye titration	Colorimetric
5	0	60	71
5	1	57	71
5	3	20	73
5	5	0	69
6	0	60	65
6	1	58	65
6	3	37	68
6	5	3	61
8	0	69	75
8	5	6	81
8	8	0	77
9	5	13	83
9	11	0	79
9	15	0	65

to check on this point, 2 samples of guava juice were assayed by the dye-titration method both before and after reduction with H_2S, as well as by the colorimetric method. Assays were made on the 2 samples on the day the bottles were opened and after 3 and 4 weeks' storage in the refrigerator. The results are shown in table 2.

The data show that these samples did contain some dehydroascorbic acid as determined by the dye-titration method, but

the total ascorbic acid measured by this method was considerably less than that found by the colorimetric method. Also there was a loss of total ascorbic acid during storage as measured by the dye-titration method, whereas the colorimetric method indicated little or no loss.

TABLE 2

Comparison of values for total ascorbic acid in guava juice as determined by the dye-titration and colorimetric methods. All values are in mg/100 ml of juice.

WEEKS STORED	SAMPLE 1 [1]				SAMPLE 2 [1]			
	Dye-titration method			Colorimetric method	Dye-titration method			Colorimetric method
	Reduced	Total	Dehydro	Total	Reduced	Total	Dehydro	Total
0	27	29	2	37	61	64	3	77
3	20	24	4	30	24	41	17	79
4	16	23	7	34	5	27	22	78

[1] Sample 1 was 2 years old and sample 2 was 7 months old at the time the bottles were opened.

TABLE 3

Comparison of values by the 2 methods for firm and for soft fruits. All values are in mg/100gm of fruit.

SAMPLE		DYE-TITRATION METHOD			COLORIMETRIC METHOD
		Reduced	Total	Dehydro	Total
1	Firm	204	201	..	210
2	Firm	136	137	1	145
3	Firm	125	129	4	132
4	Soft	92	109	17	128
5	Soft	98	117	19	148
6	Soft	80	99	19	125

The same procedure was then applied to fresh ripe guavas, some of which were firm, unbruised fruit and others which were soft, overripe and slightly bruised. Three samples of firm and 3 of soft fruits were assayed. The results are shown in table 3.

In this case the 2 methods check reasonably well with fresh firm fruit, but with soft, bruised fruit the colorimetric method again gives high values. Apparently some substance occurs in old samples which reacts with the reagent in the colorimetric method but does not react with the dye. This unknown substance seems to be a derivative of ascorbic acid, for the values obtained with the colorimetric method agree fairly well with those yielded by the dye-titration method in the case of fresh samples. Also during the storage of juices the values have always been equal to or less than the original value, never significantly higher. It seemed possible, however, that the guava extracts contained some substance which prevented the quantitative reduction of dehydroascorbic acid by H_2S and that the colorimetric values were correct.

This point was checked by the use of a biological method. There were available at the time 4 subjects who had been maintained for 5 weeks on an ascorbic acid-low diet supplemented with 75 mg ascorbic acid per day. These subjects had been maintaining very constant levels of excretion of ascorbic acid for 3 weeks. Any change of intake of ascorbic acid would be expected to be very quickly reflected in the urinary excretion. Thus it should be possible to check the true ascorbic acid content of guava juice by using these subjects. They were, therefore, continued on the previous ascorbic acid-low basal diet, but given various experimental supplements. Subject 1, acting as a positive control, was given 90 mg of synthetic ascorbic acid per day instead of the previous 75 mg. Subjects 2 and 3 were given guava juice in amounts which, according to the dye-titration method, provided 75 mg of ascorbic acid per day, but which, according to the colorimetric method, provided 110 and 115 mg per day, respectively. Subject 4 was given daily supplements of a guava juice which provided 75 mg per day on the basis of the colorimetric method, but only 37 mg per day according to the dye-titration method. These new supplements were given daily over a period of 1 week and daily excretions of ascorbic

acid were determined throughout the week. The results are shown in table 4.

Subject 1 who received an additional 15 mg of synthetic ascorbic acid per day showed an increased average excretion of 7 mg per day. Of the 3 subjects (numbers 2, 3 and 4) who received their ascorbic acid in the form of guava juice, numbers 2 and 3 showed no change in excretion level and number 4 showed a sharp drop. This is exactly what would be expected on the basis of the assay values determined by the dye-titration method. On the basis of the colorimetric assay values, subjects 2 and 3 would be expected to show increased excretion values and subject 4 should show no change. These data,

TABLE 4

Effect of a change of supplements on the urinary excretion of ascorbic acid.

SUBJECT	INTAKE			AVERAGE EXCRETION			
	Previous	Experimental		Previous weeks			Experimental [1]
		Dye method	Colorimetric method	1	2	3	
	mg/day	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*
1	75	90	...	27	24	26	33
2	75	75	110	21	21	22	22
3	75	75	115	19	21	19	18
4	75	37	75	36	34	33	16

[1] Last 3 days of the period.

therefore, clearly support the assay values obtained by dye-titration and show that the colorimetric method gave false high values for the ascorbic acid content of the samples of guava juice.

Pijoan and Gerjovich ('46) have reported a similar experience with orange juice and concluded that the false high values were due to the presence of diketogulonic acid, a derivative of ascorbic acid. This is the probable explanation for the apparently high ascorbic acid values found in guava juice since these values were always related to the original ascorbic acid content. The interference of reductones and other related compounds was ruled out by using the modifications of

the Roe and Kuether method described by Penney and Zilva ('45). These authors point out that 2,3-diketogulonic acid cannot be distinguished from l-ascorbic acid by this method.

Apparently the colorimetric method must be applied with caution particularly in the case of samples which have been stored. If the object is to determine the amount of ascorbic acid which was originally present, then the colorimetric method may be the method of choice.

SUMMARY AND CONCLUSIONS

Determinations of the ascorbic acid content of stored guava juice by the colorimetric method gave values which: (1) were considerably higher than those obtained by dye-titration and (2) indicated little or no loss of ascorbic acid during storage of the juice. Samples of guava juice given to human subjects who were receiving an ascorbic acid-low diet resulted in urinary excretion values which corresponded to the assay values obtained by dye-titration.

The colorimetric method, therefore, gave false high values for the ascorbic acid content of the stored guava juice.

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THE EFFECT OF VITAMIN A DEFICIENCY UPON THE NITROGEN METABOLISM OF THE RAT

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TWO FIGURES

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The role of vitamin A in metabolism remains obscure. In order to obtain data that might bear on this problem this experiment on the changes in nitrogen metabolism of vitamin A-deficient animals was undertaken.

Sampson, Dennison and Korenchevsky ('32) reported increased nitrogen metabolism and decreased rate of gain in body weight per gram of nitrogen ingested when rats were being depleted of vitamin A, as compared with pair-fed animals receiving vitamin A. Emerique ('37) found during avitaminosis A that the nitrogen balance remained positive although progressively smaller, and concluded that the failure of protein anabolism was the cause of the eventual loss of appetite. Braman et al. ('35) observed in a paired feeding experiment lasting 84 days that on a vitamin A-deficient diet, young rats gained less live weight and less nitrogen than their controls, but were not different from the latter with respect to digestion of the diets, nitrogen excretion, energy and fat production per gram of nitrogen retained. Only borderline deficiency was established in these rats so that the food intake of the deficient group was maintained at nearly normal levels.

Sure and Ford ('42), in an extensive study of the effect of 4 B vitamin deficiencies upon the urinary nitrogen partition

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in rats, found that the excretion of ammonia, allantoin, and creatinine was significantly changed on one or more of the deficient diets examined. No data as to food intake or nitrogen balance were reported.

METHOD

Several litters of similar age and body weight were divided at weaning, with the usual precautions, into 3 groups, and placed at once upon the diets as described later. The controls were fed the basal diet *ad libitum*, as were those given the vitamin A-free diet. The restricted controls which received vitamin A were given only the amount of basal diet eaten by the vitamin A-free group, but in proportion to the body weights.

Two series of experiments were made at different levels of protein content. In the first series the basal diet (diet I), consisted (in per cent) of vitamin-free casein 18, irradiated hydrogenated cottonseed oil 5, agar 2, salts [2] 4, cornstarch 71. In the second series the basal diet (diet II) contained in per cent vitamin-free casein 22, irradiated hydrogenated cottonseed oil 5, salts [3] 2.5, cornstarch 60.5, brewers' yeast 10. The B vitamins in the first series were supplied as rice bran concentrate [4] 0.1 gm and autoclaved yeast [5] 0.5 gm per rat per day. Cod liver oil providing 100 I.U. vitamin A per 100 gm rat per day was given the control and restricted groups in both series. Diet I contained 2.4 to 2.6% N and diet II, 3.4 to 3.5%.

In the first experiment both young and adult animals were used; in the second, only young rats. The young rats were placed on the experimental diet at 21 days of age and the adult animals at 85 days of age, when they weighed approxi-

[2] Osborne, T. B., and L. B. Mendel, J. Biol. Chem., *45:* 277, 1921.

[3] Hubbell, R. B., L. B. Mendel and A. J. Wakeman, J. Nutrition, *14:* 273, 1937.

[4] This was the concentrate marketed as Galen "B" by the Galen Company, Inc., Berkeley, California.

[5] Northwestern brewery yeast autoclaved 8 hours at 15 to 18 lbs. pressure, and dried at 100°C.

mately 145 gm. The latter group, designated series IC, consisted of 20 rats from the same litters used in series IA, which were placed on the basal diet with full vitamin supplements at weaning and which, after 64 days were distributed among the 3 experimental regimes. Nitrogen balances and urinary nitrogen partitions were then recorded throughout the depletion and deficiency periods of 124 days. After 78 days, however, the deficient and restricted groups were each divided into 2 subgroups, the animals showing evidence of deficiency, series IC_1, thus being separated from those which were not yet fully depleted, series IC_2.

At the beginning and end of the experiments, representative rats of each group were fasted for 12 hours, to insure relatively empty gastrointestinal tracts, weighed, killed by gassing and the entire carcasses put through a grinder several times. Nitrogen, fat, water and ash analyses by the usual procedures were carried out immediately. The nitrogen content of the basal diet, supplements, urine and feces was determined by Kjeldahl procedure, upon aliquots of the pooled urine and washings and pooled feces of the different groups. The methods employed for the estimation of the various urinary constituents were Van Slyke manometric method ('32) for urea, the permutit and Nesslerization directions of Folin and Bell ('17) for ammonia, the method of Benedict and Franke ('22) for uric acid, Folin method ('14) for total and preformed creatinine, and, in most instances, the Handovsky modification ('14) of the Wiekowski method for allantoin. Some allantoin determinations in the first series were made by the Larson method ('32) and those in the second series were all made by the method of Young and Conway ('42).

RESULTS

Growth

The young rats on diet I grew less than those on diet II, possibly because of the lower protein and B vitamin contents of their diet, resulting in about 10% less food consumption.

The relative performances of the vitamin-deficient, restricted and control groups in the 2 experiments were similar, however, and are summarized together (table 1). The deficient young animals in both series had about 93% of the food and protein intake of the 2 fullfed controls but gained only 39% as much weight, while the restricted normal group also consumed 93% of the control intake but attained 74% of its weight increase. The decrease in weight gain was nearly directly proportional to decrease in intake in the restricted group, but was 2 to 3 times as great in the deficient group. This was similar to, but more striking than, the difference found by Braman et al. ('35) in mildly vitamin A-deficient rats.

The response of the older rats was different. During the 78 days of the preliminary depletion period, small gains were made by all the groups. In the latter portion of the period, when the depletion was evident, the pairfed rats had 70 and 74% and the deficient animals had 56 and 77% of the intake of the unrestricted controls, but both groups lost weight in about the same proportion. There seemed to be little difference between the restricted and deficient adult rats in utilization of calories and protein.

Carcass analyses

The losses in body weight during the avitaminosis were accompanied by loss of fat from the carcass and increased content of water and, in the oldest rats, increased content of protein (table 2).

Representative carcasses were analyzed in experiment IA when the rats were 52 and 73 days old. The former group gave data on the body composition of the animals when the first failure of appetite occurred in the deficient group but before any other signs of depletion were seen, and the latter furnished information on the condition when the experiment was terminated after severe avitaminosis had been established. The body composition of all the 52-day-old rats was similar, but loss of fat and small increases in water were seen in the bodies of the 73-day-old deficient animals. Similar changes

TABLE 1

Gain per gram nitrogen intake of normal, pairfed and vitamin A-deficient young and adult rats.

DIET	SERIES	AGES	NO. OF RATS	CONDITION	BODY WEIGHTS			PERIOD	N INTAKE		GAIN PER GM N EATEN
					Beginning	End	Gain		Daily per 100 gm wt.	Total	
		days			*gm*	*gm*	*gm*	*days*	*mg*	*gm*	*gm*
I	IA	36–74	11	Normal	71	159	88	38	220	9.6	9.2
			11	Pairfed [1]	64	129	65	38	205	7.4	8.8
			11	Deficient	59	93	34	38	205	5.9	5.7
II	II	31–61 or 65	11	Normal	59	165	106	30	344	11.8	9.0
			11	Pairfed	59	142	83	34	303	10.5	7.9
			14	Deficient	59	101	42	34	314	8.7	4.8
I	IC	85–164 [2]	6	Normal	146	198	52	78	125	15.8	3.3
			7	Pairfed	142	172	30	78	116	14.0	2.3
			6	Deficient	138	176	38	78	126	15.3	2.5
I	IC_1	165–181	5	Normal	198	205	6	17	127	4.3	1.4
			3	Pairfed	171	156	−15	17	88	2.4	−6.2
			3	Deficient	194	172	−22	17	71	2.1	−10.0
I	IC_2	165–240	3	Normal	224	249	25	46	144	16.5	1.5
			3	Pairfed	186	172	−14	46	106	8.4	−1.7
			3	Deficient	175	153	−22	46	111	8.4	−2.6

[1] In all cases intake of the pairfed groups was equalized per 100 gm body weight with that of the deficient group.

[2] On complete diets from weaning to 85 days of age.

were observed in the deficient young rats of experiment II. The adult rats, 178 days old when sacrificed, were found to diverge from the others in that those receiving vitamin A had considerably more fat and the deficient animals less fat than the corresponding young rats in the other 2 experiments. The nitrogen and ash contents of the deficient group were significantly greater than those of either the control or pairfed group.

TABLE 2

Composition of the carcasses of normal, pairfed and vitamin A-deficient rats.

SERIES	GROUP	AGE	NO. OF RATS	AVERAGE BODY WEIGHT	FAT (ETHER EXTRACT)	PROTEIN	WATER	ASH	REST
		days		*gm*	%	%	%	%	%
0	Normal	31	17	53	4.9	18.7	71.3	3.8	1.3
IA	Normal	52	4	111	6.1	21.3	67.3	3.8	1.5
	Pairfed	52	3	99	6.6	20.9	67.3	4.5	0.7
	Deficient	52	4	86	5.6	21.2	68.2	4.1	0.9
	Normal	73	4	146	7.4	21.1	66.6	3.9	1.0
	Pairfed	73	4	129	8.4	20.4	66.5	4.0	0.6
	Deficient	73	5	97	3.8	21.4	70.1	4.1	0.6
IC_1	Normal	178	5	208	17.7	18.3	61.1	4.2	1.3
	Pairfed	178	3	187	10.7	20.0	66.1	4.8	1.6
	Deficient	178	3	161	2.2	24.5	66.6	6.5	0.2
II	Normal	59	8	163	8.9	20.0	66.3	3.0	1.8
	Pairfed	63	9	147	7.4	20.2	65.6	3.6	3.2
	Deficient	63	11	95	2.6	20.8	69.0	3.1	4.5

The retention of food nitrogen in the carcasses of the 2 groups of young rats (table 3) indicates the same efficiency of transformation of food to body protein by the normal and pairfed rats on the 2 diets, and a lower utilization by the deficient rats in both cases, namely, 55 and 62% of the normal.

The nitrogen balances

The nitrogen balances of the young deficient rats were progressively less positive as the deficiency increased in

severity. This was due to increased urinary output since there was little change in the fecal nitrogen other than that produced by changes in the intake. Results of some of the studies are shown in table 4. The nitrogen metabolism in all series was studied until some or all of the deficient group had succumbed. The average retention of the young rats on diet II was 30 to 50% greater in all cases than in the corresponding groups on diet I, but the retention of the pairfed group was significantly greater in both series than that of the deficient animals. The deficiency state reached by the rats in series IA was more severe than that shown by the animals in series

TABLE 3

Retention of food nitrogen in the carcasses of normal, pairfed and vitamin A-deficient rats.

SERIES	GROUP	AGE	NITROGEN CONTENT OF CARCASS			TOTAL NITROGEN INTAKE	RETENTION OF NITROGEN OF INTAKE
			At beginning	At end	Gain		
		days	*gm*	*gm*	*gm*	*gm*	%
IA	Normal	52–73	3.78	4.93	1.15	5.3	22
	Pairfed	52–73	3.31	4.21	0.90	4.1	22
	Deficient	52–73	2.91	3.31	0.40	3.3	12
II	Normal	31–59	1.77	5.12	3.35	11.5	29
	Pairfed	31–63	1.77	4.32	2.55	9.2	28
	Deficient	31–63	1.77	3.30	1.53	8.4	18

II, and the average nitrogen retention for series IA was also less.

No clear change in the nitrogen balance was observable in any of the adult deficient rats other than that simultaneously shown by their pairfed mates, even though all the deficient adult animals died of the deficiency. The rapidity of the onset of terminal changes appeared to influence the large nitrogen losses of series IC_1 as compared with IC_2. Short periods of 8 and 6 days, in which the deficient and pairfed adult rats had exactly equal intakes, indicate the relatively small advantage in nitrogen retention of the rats which received vitamin A.

The lesser effect of the deficiency upon the nitrogen retention of the young rats fed the higher protein diet as well as upon the adult animals on the lower level of intake may indicate some role of vitamin A in the metabolism of the amino acids of borderline indispensability, for example, arginine to the young and histidine to the older animals.

TABLE 4

The effect of vitamin A deficiency and food restriction upon the nitrogen retention of rats.

SERIES	GROUP	NO. OF RATS	AGE	AVERAGE BODY WT.	BALANCE PERIOD	N IN MG PER 100 GM BODY WEIGHT PER DAY			
						Intake	Urine	Feces	Balance
			days	*gm*	*days*				
IA	Normal	11	36–66	106	30	220	103	15	100
	Pairfed	11	36–74	98	38	205	117	16	72
	Deficient	11	36–74	83	38	205	154	18	33
II	Normal	11	31–65	113	34	344	186	28	130
	Pairfed	11	31–69	103	38	303	178	25	100
	Deficient	14	31–69	87	38	314	194	34	86
IC	Normal	7	86–164	166	78	125	106	16	3
	Pairfed	6	86–164	156	78	111	110	14	—13
	Deficient	7	86–164	158	78	126	117	16	—7
	Normal	6	94–102	162	8	125	106	15	4
	Pairfed	6	94–102	153	8	126	106	15	5
	Deficient	7	94–102	155	8	125	122	14	—11
IC_1	Normal	5	165–182	205	17	127	106	20	1
	Pairfed	3	165–182	166	17	87	90	13	—16
	Deficient	3	165–182	181	17	71	84	11	—24
	Pairfed	3	170–176	162	6	63	77	11	—25
	Deficient	3	170–176	178	6	64	85	8	—29
IC_2	Normal	3	165–210	222	46	139	106	21	12
	Pairfed	3	165–210	181	46	106	98	13	—5
	Deficient	3	165–210	166	46	113	109	13	—9

Urinary nitrogen partition

As shown in table 5, although the level of nitrogen excretion was elevated in all the deficient and pairfed rats, there was no significant change in the urinary nitrogen partition, due

TABLE 5

Daily average urinary nitrogen partition of normal, pairfed and vitamin A-deficient rats.

SERIES, AGE (DAYS) AND GROUPS	URINARY N	UREA N		AMMONIA N		ALLANTOIN N		URIC ACID N		CREATININE N		CREATINE N		UNDETERMINED N
	mg [1]	mg	% [2]	mg	%	mg	%	mg	%	mg	%	mg	%	%
IA														
35–73														
Control	105	93	90.2	2.2	1.8	3.6	3.5	0.24	0.24	1.1	1.1	3.1	3.0	0.2
Pairfed	119	104	90.5	1.8	1.6	4.0	3.4	0.31	0.27	1.1	0.9	3.3	2.9	0.4
Deficient	154	140	90.7	1.8	1.4	5.5	3.6	0.32	0.21	1.3	0.9	3.8	2.4	0.8
II														
31–69														
Control	186			7.8	4.2	8.0	4.3	0.43	0.23					
Pairfed	178			9.9	5.5	8.0	4.8	0.39	0.23					
Deficient	194			16.6	5.8	8.4	4.2	0.46	0.24					
IC														
86–164														
Control	107	99	92.5	0.9	0.9	2.5	2.4	0.15	0.14	1.1	1.0	1.7	1.6	1.5
Pairfed	109	100	91.8	1.0	0.8	2.1	1.9	0.16	0.15	1.1	1.0	1.8	1.6	2.8
Deficient	117	108	92.5	0.8	0.9	2.3	1.9	0.15	0.17	1.1	0.9	2.0	1.7	2.0
IC_1														
165–182														
Control	105	99	94.0	0.6	0.6	2.4	2.3	0.12	0.11	1.1	1.0	1.0	1.0	1.0
Pairfed	92	84	90.8	0.9	1.0	2.9	3.2	0.13	0.14	1.2	1.3	1.3	1.4	2.2
Deficient	82	74	90.5	1.1	1.3	3.6	4.4	0.12	0.14	1.1	1.4	1.4	1.6	0.7
IC_2														
165–210														
Control	104	99	94.5	0.4	0.4	2.6	2.5	0.10	0.10	0.8	0.8	1.5	1.4	0.3
Pairfed	96	88	91.5	0.6	0.6	3.4	3.5	0.13	0.13	0.9	0.9	1.5	1.6	1.8
Deficient	106	98	92.0	0.8	0.7	3.7	3.5	0.13	0.12	1.0	0.9	1.8	1.7	1.1

[1] In all cases this represents mg N per 100 gm body weight per day.

[2] Of total urinary nitrogen.

either to starvation or to the deficiency. In series IC_1, representing the most advanced and in some cases premortal changes, a rise in the excretion of ammonia and allantoin and a larger drop in urea excretion were manifested by the vitamin-deficient as compared with the pairfed groups. This was not true of the more prolonged terminal stages shown in series IC_2.

Ammonia nitrogen was found in somewhat increased amount only in the premortal excretion of the deficient and pairfed adult animals. The ammonia output remained at a low level, about 1% of total nitrogen, throughout all the balances on

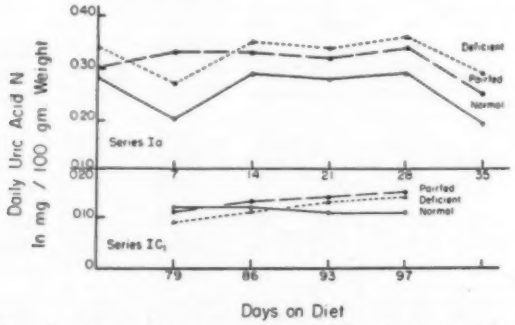

Fig. 1 The uric acid excretion of normal, pairfed and vitamin A-deficient young (series IA) and adult (series IC) rats.

diet I, but was increased to from 4 to 7% on the higher protein diet II. Urea was not determined in the latter series.

Preformed creatinine remained constant at about 1.0 mg per 100 gm body weight in all the groups, young and old. The creatine output was about twice as large in the young as in the older animals, in proportion to total nitrogen output. This is the usual result of decreased food intake.

The uric acid excretion was twice as great in the young as in the older animals but there was no change which could be ascribed to either starvation or the deficiency in any of the series, except for the slight premortal rise noted in both the deficient and pairfed groups of series IC_1 (fig. 1).

The allantoin excretion was also greater in proportion both to body weight and to total urinary nitrogen in the young than in the older rats. Both young and adult deficient animals excreted more allantoin than the pairfed or normal rats from the time that the deficiency became manifest, and particularly in the premortal period (fig. 2). This is not clearly shown in the daily averages compiled for the entire period of observa-

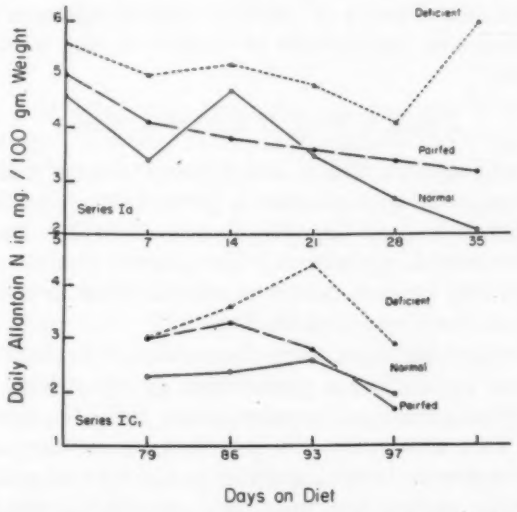

Fig. 2 The allantoin excretion of normal, pairfed and vitamin A-deficient young (series IA) and adult (series IC_1) rats.

tion, in which the premortal changes are masked. On diet II in which the deficiency was less severe than on diet I, the increased allantoin output of the deficient rats was not observed. This may be due to the more generous supply of necessary amino acids available, to the relatively mild deficiency, or to both of these factors. In several other experiments with the lower protein diet and severely deficient young animals the increased allantoin excretion was repeatedly observed. Sure and Ford ('42) reported only decreases in both

allantoin and uric acid in all their B vitamin-deficient rats, but again the values quoted were averages without separate report on terminal figures.

If allantoin and uric acid are products of essential tissue degradation, an accelerated rate of such breakdown occurred in both the vitamin A-deficient rats and their pairfed controls in the final stages of the deficiency. The greater excretion of allantoin by the deficient group might mean that purine or pyrimidine constituents of certain enzyme systems may become unusable in the absence of vitamin A and be discarded as allantoin.

Effect of level of dietary protein

The young rats on diet II which received nearly 40% more protein than did those on diet I, grew more than the latter but utilized their food for growth less efficiently, whether or not they received vitamin A. The higher protein fed rats which received vitamin A stored considerably more fat than did those on the lower protein diet.

The nitrogen balances were also about 40% greater in the higher than in the lower protein-fed groups which received vitamin A but were much greater, about 150%, in the deficient animals. This was due to the fact that the urinary excretion of nitrogen was 50 to 80% greater in the normal and pairfed high protein groups but only 26% greater in the deficient animals, than in the corresponding lower protein-fed rats. It must be remembered, however, that the deficiency was more severe in the latter series.

The proportion of nitrogen excreted as uric acid and allantoin was not changed by the difference in level of protein intake, but the absolute and relative amount of urinary ammonia nitrogen was raised in the group fed the more protein-rich diet.

It appears that the catabolic processes of the protein metabolism were abnormally stimulated in the vitamin A-deficient growing organism, beyond that which might have been expected due to decreased food intake. The qualitative nature

of the resulting tissue breakdown or failure of tissue growth was not different from that produced by partial inanition alone, with the possible exception of the purine metabolism. This confirms the findings of Sampson and Korenchevsky ('32).

Since the nitrogen metabolism of the adult animals was not affected by the deficiency except in the premortal stage, it may be concluded that vitamin A is essential for tissue growth but not for maintenance.

SUMMARY

Young and adult rats from the same litters were divided into 3 groups, vitamin A-deficient, pairfed and fullfed on a diet containing 18% casein, and their nitrogen metabolism studied for 30 to 124 days. Another group of young rats was placed on a 22% casein diet and was similarly studied.

As the deficiency progressed, growth per gram of protein eaten declined in the deficient young rats to 65% of that of the pairfed groups. The urinary nitrogen increased and the nitrogen balance was decreased to a greater degree in the deficient than in the pairfed young animals. But in the adult group weight changes and nitrogen balances were alike in the pairfed and deficient rats.

The urinary nitrogen partitions were essentially alike in the pairfed and deficient groups except in the severely deficient and premortal periods when the allantoin output of the deficient animals was increased above that of the pairfed litter mates.

The composition of the carcasses of the fullfed, pairfed and deficient young rats remained constant except for the fat content which was diminished in the latter group with corresponding increase in water. As shown by carcass analysis, the retention of food nitrogen by the pairfed and fullfed young rats was the same in both series, but that of the deficient groups was only 55 and 62% of these values.

The deficiency state, as distinct from the accompanying inanition, decreased the utilization of nitrogen in the young

animals without affecting the character of the nitrogen metabolism except in the terminal period. The adult deficient and pairfed groups exhibited no differences in nitrogen metabolism. Vitamin A may therefore be considered essential for the growth of tissue protein but not for its maintenance.

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NITROGEN METABOLISM OF THE NORMAL AND THE VITAMIN A-DEFICIENT RAT AS AFFECTED BY THYROID ADMINISTRATION

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ONE FIGURE

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Possible interrelations between vitamin A and thyroid function have been the subject of numerous investigations in recent years. In a review, Drill ('43) summarized the status of the question as controversial.

Chevallier and Baert ('34) found that the basal metabolic rate of rats tended to rise on vitamin A depletion and to fall below normal when large doses were given. Sadhu and Brody ('47) have also observed the latter phenomenon. Abelin ('35) reported that vitamin A could reduce the high basal metabolic rate produced in rats given toxic doses of thyroxine. Belasco and Murlin ('40) found that vitamin A and ascorbic acid reduced to some extent the hypermetabolism associated with hyperthyroidism. Sheets and Struck ('42) saw no decrease in the metabolic rate of thyroid-treated rats due to administration of large doses of vitamin A.

That thyroxine increases the utilization or decreases the storage of vitamin A is the view held by a number of investigators, including Euler and Klussmann ('32) and Fasold and Peters ('33). However, Logaras and Drummond ('38) and Baumann and Moore ('39) came to the opposite conclusion.

Vitamin A deficiency has usually been found to produce hypertrophy of the thyroid although some investigators have reported no effect. Coplan and Sampson ('35) noted a sex difference in rats in that the deficiency caused thyroid hypertrophy in the female but atrophy in the male. That there is an increased need for vitamin A in hyperthyroidism has been reported by many, including Drill ('43), Belasco and Murlin ('40) and Sure and Buchanan ('37).

The fact that the relationship of the thyroid to vitamin A activity is not clear appeared to warrant the present study. Furthermore, it seemed worthwhile to investigate in more detail the hypothesis that the requirement of vitamin A is increased in hyperthyroidism. If this is true one might expect thyroid-treated animals on vitamin A-deficient diets to succumb to vitamin A deficiency readily or to be exceedingly sensitive to hyperthyroidism so that thyrotoxicosis would speedily become prominent and severe. No studies of this problem appear to have been made.

The nitrogen metabolism of vitamin A-deficient young rats previously examined (Brown and Morgan, '48) by the methods used in the present study had been found to be markedly influenced by the deficiency as well as by the accompanying reduction in food intake. The addition of hyperthyroidism to the deficiency as well as to the inanition state might be expected to produce a further disturbance of the nitrogen metabolism which should be cumulative, if there is neither antagonism nor coördination of the thyroid and vitamin A functions. The changes in nitrogen metabolism of normal and vitamin A-deficient rats with and without thyroid medication were therefore used as an index of the interrelation.

METHOD

The basal diet was that designated diet II in the previous report (Brown and Morgan, '48). This diet provided ample amounts of iodine. Cod liver oil, providing 100 I.U. of vitamin A per 100 gm body weight per day was given to the non-

deficient rats, and desiccated thyroid,[1] 100 mg per 100 gm body weight per day administered by mouth to the hyperthyroid groups. Young rats from 20 litters were placed at weaning on the diet and were grouped so that weight, sex and litter origins were equally comparable in the 6 groups used. These groups were (1) normal, that is, receiving vitamin A and with no food restriction, (2) restricted or pairfed, receiving vitamin A but with food intake restricted to that of the deficient group in proportion to body weight, and (3) deficient, without vitamin A and not restricted as to food. Three additional groups on these regimes were given the desiccated thyroid. The males and females of each group were studied separately, so that in all 12 experimental groups were formed.

Soon after they were weaned, representative rats were sacrificed and moisture, ash, protein and fat determined in the carcasses. Similar analyses were carried out on all carcasses at the end of the experiments.

Urine and feces collections were made (a pooled sample for each group) for periods of 3 or 4 days consecutively for 34 to 38 days. Observations were made on the nitrogen balance, and on the excretion of ammonia, uric acid and allantoin. The methods employed were the same as those used previously (Brown and Morgan, '48), the allantoin being determined by the directions of Young and Conway ('42).

The experiments were terminated soon after all the rats of the groups restricted in intake and given thyroid had died. At this time the vitamin A-free groups were not as deficient as were the animals at the termination of studies in our earlier experiments. Less noticeable changes in all factors previously noted in vitamin A deficiency were therefore to be expected. No rats had demonstrated extreme avitaminosis and none had succumbed to the deficiency when it was uncomplicated by thyroid administration. Between the thirtieth and thirty-eighth day of the experiment, 4 males and 1 female

[1] Desiccated thyroid, U.S.P.

of the deficient group given thyroid had died, as had also 2 females and 1 male of the normal group given thyroid.

RESULTS

Growth

The growth of the groups is shown in figure 1. It will be noted that only in the thyroid-treated animals which were restricted as to food intake was there any consistent loss of weight during the period of the experiment. However, the gains made by the normal, pairfed and deficient groups of

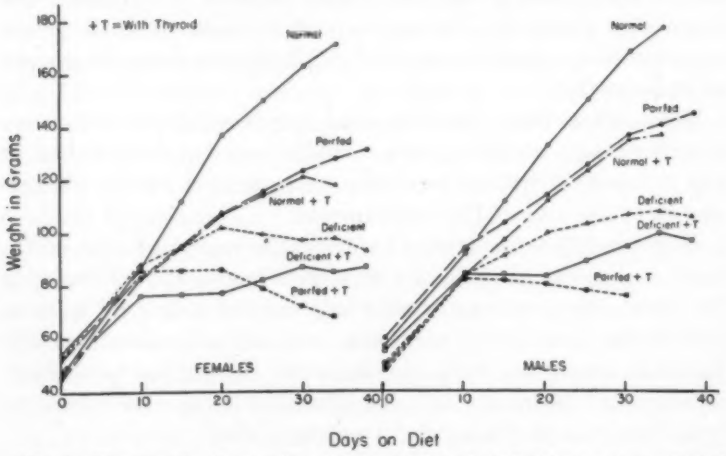

Fig. 1 The growth of normal, pairfed and vitamin A-deficient rats as affected by thyroid administration.

both sexes were always greater than those made by the corresponding hyperthyroid animals. There was only a small difference, however, between the deficient groups with and without thyroid.

The utilization of food nitrogen for growth (table 2) was decreased by the vitamin A deficiency as well as by thyroid treatment. It was evident that food restriction had little effect on protein utilization when vitamin A was present, but that the vitamin-deficiency markedly reduced utilization,

particularly in the females. The thyroid treatment only slightly affected the deficient females, but more seriously the deficient males. When these values are compared with the utilization by the normal groups either with or without thyroid, it is evident that the vitamin deficient rats were less affected by the thyroid treatment than were the normal unrestricted groups. In fact, the deficient females were benefited by the treatment when compared with the control thyroid-treated group, and the males were affected exactly as much as were their normal controls.

The most severe growth failures were seen in the food-restricted groups given thyroid.

So far as utilization of protein for growth is concerned, the amount of thyroid medication employed reduced by 54 and 36% the efficiency of normal well-fed female and male rats, by 16 and 33% that of vitamin A-deficient animals, and by 83 and 84% that of normal but food-restricted animals. In the female animals the deficiency apparently decreased the thyroid toxicity, and in the males it had little effect upon that condition. On the other hand, the thyroid seemed to nullify the deficiency effects in the females but to produce no change in the males. Food restriction alone affected utilization relatively little but depressed it severely when thyroid was given.

Administration of this amount of thyroid induced marked increases in food intake, while vitamin A deficiency alone caused somewhat decreased appetite. Vitamin A-deficient rats when given thyroid showed no inanition tendency, however, but ate as much or more than did the normal rats. An interesting finding here is that the usual and general trend of decreased intake per 100 gm weight as the animals became older did not hold either in the control group receiving thyroid or in the vitamin A-free rats given thyroid.

Sampson and coworkers ('32) reported a decrease in efficiency of food utilization for growth when rats were being depleted of vitamin A. This observation was verified in our results. Our hyperthyroid animals were likewise inefficient in

protein utilization. This was observed also by Koger, Hurst and Turner ('42).

Carcass analysis

Since the nitrogen retention of the thyroid-treated rats was in some cases greater than that of the non-hyperthyroid animals, analysis of the carcasses seemed indicated. In table 1

TABLE 1

Composition of the carcasses of normal, vitamin A-deficient and pairfed rats as affected by thyroid administration.

GROUP		AGE	NO. OF CARCASSES	AV. BODY WEIGHT	ETHER EXTRACT	PROTEIN (N×6.25)	WATER	ASH	REST
		days		*gm*	%	%	%	%	%
Young, representatives of litters		31	17	56	4.9	18.7	71.3	3.8	1.3
Normal	♀	65	4	163	9.2	19.6	65.9	3.1	2.2
	♂	65	4	163	8.6	20.3	66.4	2.8	1.9
Normal with thyroid	♀	65	3	117	5.2	18.4	70.1	3.4	2.9
	♂	65	4	140	4.7	19.5	69.4	4.0	2.4
Deficient	♀	69	6	90	2.6	20.8	69.1	3.0	4.5
	♂	69	5	101	2.6	20.8	68.8	3.2	4.6
Deficient with thyroid	♀	69	5	87	3.6	20.3	70.2	3.6	2.3
	♂	69	4	98	3.1	20.7	68.2	3.8	4.2
Restricted	♀	69	5	136	7.3	19.8	65.5	3.0	4.4
	♂	69	4	159	7.4	20.5	65.7	4.2	2.2
Restricted with thyroid	♀	61	5	63	0.9	21.1	70.4	4.6	3.0
	♂	61	7	66	0.9	19.5	72.2	4.4	3.0

the results are shown. Both the vitamin A deficiency and the thyroid treatment separately reduced the body fat and increased its water content, but together had less effect. There was no definite change in any case in the protein content.

The proportion of moisture in the carcasses of normal rats decreases as the animal ages. Animals given thyroid and those deficient in vitamin A had moisture content resembling

that of young weanling rats, with little difference between males and females. Thyroid administration caused a more marked increase in moisture content of the tissues of both normal and pairfed animals than in those of the deficient animals.

By calculation from the composition of these carcasses and of those of representative young rats at 31 days of age, at the beginning of the experiment, the amount of food protein retained can be obtained (table 2). The retention of food nitrogen in the carcasses of the normal and restricted groups was nearly the same but in the vitamin A-deficient group it was only a little more than half as much. All of the thyroid-treated groups retained less food protein in the carcasses than did their controls. The hyperthyroid females on normal and deficient diets retained the same proportion of intake, but the hyperthyroid deficient males retained slightly less than the corresponding animals on the normal diet. The hyperthyroid animals which were restricted in intake retained only 5 or 6% of the intake. Thus, the thyroid treatment decreased the protein-building ability of the normal females by about 60%, but of the deficient females by only 23%, and of the pairfed females by 76%. In the deficient and also the control males the thyroid depressed tissue production by the same amount, 40%, but in the pairfed males by 82%.

Judged by the amount of retention of food nitrogen in the carcasses, the hyperthyroid condition affected all the males similarly, regardless of vitamin A status, but depressed the normal females more severely than the vitamin deficient females. The deficiency alone also reduced the protein retention in both sexes, but the combined hyperthyroid and deficiency states produced reduction only in the males. Insofar as the inanition effect could be judged, it appeared to produce no significant loss of efficiency, but combined with thyroid administration, the pairfed hyperthyroid animals being compared with normal hyperthyroid rats, it caused 50 and 70% loss of tissue formation. The greater depression of the growth of males than females produced by hyperthyroidism in the

TABLE 2

Gain in weight, food intake and retention of nitrogen in carcasses of normal, pairfed and vitamin A-deficient rats as affected by thyroid administration.[1]

GROUP	NO. OF RATS	BODY WEIGHTS			PERIOD	NITROGEN INTAKE		GAIN PER GM NITROGEN EATEN	NITROGEN CONTENT OF CARCASSES			FOOD N RETENTION IN CARCASSES
		At beginning [2]	At end	Gain		Total	Per day		At beginning	At end	Gain	
Females		*gm*	*gm*	*gm*	*days*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	%
Normal	6	46	171	125	34	13.7	0.40	9.1	1.38	5.37	3.99	29
Hyperthyroid normal	7	52	118	66	34	15.5	0.46	4.2	1.56	3.47	1.91	12
Deficient	10	53	93	40	38	10.4	0.27	3.8	1.59	3.10	1.51	14
Hyperthyroid deficient	6	48	87	39	38	12.0	0.31	3.2	1.44	2.83	1.39	11
Pairfed	6	45	131	86	38	11.0	0.30	7.8	1.35	4.15	2.80	25
Hyperthyroid pairfed	7	54	68	14	34	10.5	0.31	1.3	1.62	2.30	0.68	6
Males												
Normal	5	55	177	122	34	14.4	0.42	8.4	1.65	5.75	4.10	28
Hyperthyroid normal	6	49	137	88	34	16.2	0.48	5.4	1.47	4.27	2.80	17
Deficient	6	48	101	53	38	9.6	0.25	5.5	1.44	3.36	1.92	20
Hyperthyroid deficient	8	55	97	42	38	12.7	0.33	3.7	1.65	3.21	1.56	12
Pairfed	5	57	145	88	38	11.0	0.30	8.0	1.71	4.76	3.05	28
Hyperthyroid pairfed	10	50	63	13	30	9.6	0.32	1.3	1.50	1.97	0.47	5

[1] 100 mg dried thyroid per 100 gm body weight per day.
[2] At 31 days of age.

absence of vitamin A was also seen by Logaras and Drummond ('38).

Nitrogen retention

The retentions of nitrogen, as measured by the balance method, by the normal and pairfed female rats during the later period of observation were alike, but that of the deficient group was much reduced. The deficient male rats under similar circumstances retained very slightly less nitrogen than their pairfed control groups (table 3).

TABLE 3

The nitrogen metabolism of normal, vitamin A-deficient and pairfed rats with and without thyroid, from the nineteenth to the thirtieth day on the diet

GROUP	AVERAGE WEIGHT	NITROGEN					RETENTION OF NITROGEN OF INTAKE
		Intake	Urinary	Fecal	Retained	Total retained	
Females	*gm*	*mg/100 gm body weight/day*				*gm*	%
Normal	151	274	175	30	69	1.25	25
Normal with thyroid	114	443	292	58	93	1.27	21
Deficient	88	262	205	31	26	0.27	10
Deficient with thyroid	85	443	285	67	91	0.93	21
Pairfed	125	255	178	27	50	0.75	20
Pairfed with thyroid	75	333	294	58	—19	—0.17	—6
Males							
Normal	156	293	157	29	107	2.00	36
Normal with thyroid	127	418	249	78	91	1.39	22
Deficient	105	245	169	29	47	0.59	19
Deficient with thyroid	93	440	247	75	118	1.32	27
Pairfed	134	227	155	23	49	0.79	21
Pairfed with thyroid	82	291	306	65	—80	—0.79	—27

All the hyperthyroid rats without vitamin A had the same nitrogen intakes and the same or better relative retentions as compared with the corresponding groups which received the vitamin. It appeared that in both non-thyroid and thyroid-treated animals the vitamin deficiency had little deleterious influence on the nitrogen metabolism of males but depressed retention in non-thyroid treated females. The thyroid medication exerted a favorable effect in both sexes upon the nitro-

[illegible] mg dried thyroid per 100 gm body weight per day.
[illegible] At 31 days of age.

gen metabolism of the vitamin deficient animals. Thus, it is evident that both the thyroid administration and the vitamin deficiency state produced decreased nitrogen balances relative to intake, except when the 2 conditions were present together. Food restriction lowered the nitrogen retention in all cases, and combined with thyroid treatment led eventually to negative balances.

The thyroid-treated groups, both with and without vitamin A, were able to increase their intake sufficiently to maintain more positive nitrogen balances than the corresponding groups not receiving thyroid. Since the corresponding non-thyroid treated females, pairfed and normal, had more than double the nitrogen retention of the deficient group, one might well question that thyroid in the amount used and vitamin A are antagonistic in their effects. The larger nitrogen retentions in the deficient thyroid-treated groups may have been merely the result of the huge intakes. Yet, the fact that during the last several balances the hyperthyroid vitamin A-deficient groups retained as much nitrogen as the similar groups which received vitamin A is suggestive of a sparing effect due to the deficiency. The intake of these groups happened to be quite similar.

Johnston and Maroney ('39) with children as subjects found that administration of small doses of thyroid increased nitrogen retention while large doses reversed this effect. In severe hyperthyroidism positive nitrogen balances were obtained on high protein intake. Johnston ('41) reported that in girl patients resting in bed a small dose of thyroid stimulated appetite and allowed greater consumption of food so that the detrimental effect on nitrogen balance was avoided. Our rats reacted similarly to these human subjects.

Our results confirm also those of a limited experiment of Terroine and Babad ('39) using 4 rats. They reported that intraperitoneal injection of thyroxine into rats was followed by appetite increases so great that much more nitrogen was absorbed, but in spite of increased nitrogen retention there was a diminution in the rate of weight increase. In our ex-

periments the group of vitamin A-free animals given thyroid consumed larger quantities of food than either the deficient or normal animals; they retained more nitrogen in the latter part of the period yet did not attain as great weight. Similar comparisons can be made for the control groups with and without thyroid administration, where it will be noted that those which received thyroid during the period of observation ate much larger amounts of food and retained more nitrogen yet failed to gain as much weight as the control groups.

Urinary nitrogen partition

Thyroid treatment during the latter part of the period when the vitamin deficiency was apparent, increased the urinary nitrogen in control, pairfed and deficient females by about the same amount, namely, 67, 65 and 61%, respectively, but in the males 59, 97 and 46%. The output of ammonia nitrogen in proportion to total urinary nitrogen was apparently not significantly affected by thyroid, although there was some tendency to an increase with thyroid treatment. The excretion of uric acid was strikingly constant in all groups in proportion to total nitrogen output. The proportion of allantoin nitrogen in the urine was lower in all thyroid-treated animals than in their controls, but this lowering was least pronounced in the deficient groups (table 4). None of these changes are of the magnitude found by Sure and his associates ('41) in a study of much more heavily thyroxine-treated young rats, but for the ammonia and allantoin at least, are in the same direction as the changes he reported.

The lack of vitamin A produced no appreciable change in the qualitative character of the nitrogen metabolism of our rats, insofar as this was indicated by the excretion of ammonia, uric acid and allantoin.

By the usual criteria of nitrogen metabolism, that is, utilization of food protein for growth and for production of tissue protein, amount of nitrogen retention, and urinary ammonia and purine output, the mild hyperthyroidism to which these

animals were subjected produced unfavorable effects in the normal fullfed and pairfed groups but much less severe depression in the vitamin A-deficient animals, especially the females. The vitamin A requirement was not increased in the hyperthyroid deficient groups, judged by these criteria, although the level of metabolism was obviously notably raised.

TABLE 4

Daily average urinary nitrogen partition of normal, pairfed and vitamin A-deficient rats as affected by thyroid administration, from the nineteenth to thirtieth day on diet.

GROUP	URINARY N	AMMONIA N		ALLANTOIN N		URIC ACID N	
Females	mg [1]	mg	% [2]	mg	%	mg	%
Normal	175	6.8	3.8	6.3	3.6	0.36	0.20
Normal with thyroid	292	13.2	4.5	7.9	2.8	0.59	0.21
Deficient	205	10.3	4.8	7.8	3.8	0.45	0.22
Deficient with thyroid	285	29.0	10.4	9.3	3.3	0.58	0.20
Pairfed	178	9.5	5.4	6.6	4.2	0.34	0.19
Pairfed with thyroid	294	15.8	5.3	9.6	3.3	0.57	0.19
Males							
Normal	157	5.9	3.6	5.9	3.8	0.33	0.21
Normal with thyroid	249	16.2	6.5	7.4	2.9	0.48	0.19
Deficient	169	13.4	8.4	6.7	4.1	0.36	0.22
Deficient with thyroid	247	23.0	9.5	8.1	3.6	0.53	0.22
Pairfed	155	12.1	7.5	6.4	4.3	0.32	0.21
Pairfed with thyroid	306	24.0	7.6	8.8	2.9	0.55	0.18

[1] In all cases per 100 gm body weight.

[2] Of total urinary nitrogen.

At the same time, since the body weights of all of these hyperthyroid animals were lower at all times than those of the untreated groups, the amount of vitamin A available in proportion to body weight must have been larger in the former than in the latter. This would support the assumption often put forth that vitamin A requirement is dependent upon body weight and independent of calorie output.

Since the marked decrease in nitrogen utilization caused by vitamin A deficiency in females was counterbalanced by the thyroid influence, it might be assumed that in the female the deficiency reduced thyroid activity, as suggested by the work of Coplan and Sampson ('35). No such counterbalancing by thyroid feeding was seen in the males, indicating possibly in this sex no specific effect of vitamin A deficiency on the thyroid gland.

SUMMARY

The nitrogen metabolism of young rats fed a purified diet containing 22% casein, with or without vitamin A and with or without administration of 100 mg desiccated thyroid per 100 gm body weight per day was observed for 30 to 38 days.

The utilization of nitrogen for growth was depressed by both the vitamin A deficiency and the thyroid treatment, more severely in the females than the males, but there was no cumulative effect when the 2 conditions were present together. Instead, the use of protein by the deficient animals, as shown by increase in carcass nitrogen and by growth, was less affected by the thyroid than was that of their normal controls.

Nitrogen retention during the later period of the deficiency was depressed by thyroid treatment in the fullfed and pairfed normal animals but improved in the deficient groups.

There was little change in the ammonia or uric acid fraction of the urinary nitrogen in any case, but a small decrease in allantoin excretion was noted in the hyperthyroid rats which was less pronounced in the deficient groups.

No increase in the vitamin A requirement resulted from this amount of thyroid treatment in either sex, but instead there may have been a reduction in the requirement of the females. No relationship is indicated therefore between total metabolism and vitamin A requirement as shown by nitrogen exchange.

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THE ABSORPTION OF IRON FROM BEEF BY WOMEN

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Since the introduction of the theory that only a small proportion of the iron in the diet is absorbed, a number of experiments have been carried on to gather information on the actual percentage of iron which is absorbed under various conditions. Most of these studies have been carried out on animals. Human subjects on diets high in cereal content have been studied by Widdowson and McCance ('42), who found that when refined flour was used, women absorbed about 12.5% of the iron of the diet. The only other studies set up to find the percentage of iron absorbed by normal human subjects have been made with the use of radioactive iron. In these investigations iron salts rather than foods have been used. By this means normal men, children, pregnant women and normal women have been studied. The studies including normal women were made by Dubach et al. ('48) who found absorptions of from 1 to 10%. The present study was designed to contribute more information about the percentage of iron absorbed by normal women. At the same time material was collected for a study of the iron requirement which will be reported later.

The question of the method to employ was the first consideration. In recent years considerable experimental work has

accumulated which shows that iron is either not secreted or is secreted in only minute amounts from the blood plasma into the intestinal tract. Some iron may be added to the food-residue as it passes through the gastrointestinal tract due to sloughed-off cells and digestive secretions. The amount so picked up is not known; it may be even less than 0.1 mg and it is certainly never more than 0.5 mg. If the amount is assumed to be so small as to be negligible, the difference between the iron in the feces and that in the food gives the amount absorbed. This was the method employed by Widdowson and McCance ('42) whose main purpose was to compare refined and whole cereals rather than to obtain a value for the percentage of absorption. While the assumption may be valid, a method for determining absorption which would eliminate any effect of "picked-up" iron seemed preferable. The method decided upon was as follows: (1) To administer a basal diet containing an amount of iron just sufficient to cover the subject's needs; (2) to find the excretion in the feces on that diet; (3) to continue the basal diet and add the food to be tested; and (4) to determine the increase in intake due to the test-food, and the increase in fecal iron. The increases in the fecal iron would be due to the iron in the test-food that was not absorbed. The amount that was absorbed could be found by difference. This method is similar to one used by Kinsman et al. ('39) in the study of calcium absorption.

For the test-food, beef was chosen as it is used commonly in many diets and is high in iron. Furthermore, most of the previous work had indicated the iron of beef to be well absorbed. The earliest study on the absorption of iron from beef, one that did not agree with later work, and one in which rats were used as subjects, was made by Sherman, Elvehjem and Hart ('34) who concluded that the iron of beef was poorly absorbed. Later, Oldham ('41) tested cooked and uncooked beef and found that the iron of beef was as well absorbed as $FeCl_3$ if the beef was cooked. Recently, Pye and MacLeod ('46) found that 32% of the iron of dried beef muscle was absorbed by growing rats. Oldham, Schlutz and Morse ('37)

found that the iron from beef was well absorbed by an infant. Leverton, who conducted a study on young women in 1941, found very high absorptions of beef-iron when the beef was fed after a long period on a depletion diet.

PROCEDURE

The subjects were 5 young women 18 years of age who were in excellent health. The weight (in pounds) and height (in inches) for subjects A, B, C, D and E, respectively, were as follows: 147¼ and 64¼; 139¾ and 63¾; 155 and 64¼; 138¼ and 64½; and 148 and 68¾. Subject E was 4 to 5 inches taller than the others.

On the basis of Leverton's ('41) observations, 7 mg of iron was selected as the lowest intake of iron which would be adequate to cover the needs of the subjects who were placed on a basal diet of that iron content for 10 weeks. The first 2 weeks constituted a period of adjustment during which some minor changes in the diet but no fecal collections were made. During the next 4 weeks the basal diet alone was fed and during the last 4 weeks ground beef (⅓ fat, ⅔ lean meat) containing 3.4 mg of iron per day was added. This was served as 2 cakes of 100 gm each, 1 at breakfast and 1 at lunch. All the beef for the entire period was ground at 1 time, well mixed, made into 100 gm cakes and frozen.

The basal diet was adequate in all respects. It contained a quart of milk, an egg, 1 serving of meat, an average of more than 2 servings of vegetables a day besides potatoes and more than 2 servings of fruit a day. To avoid a high phytic acid content the diet contained no whole cereals, shelled beans, nuts or cocoa and only 1 serving of chocolate a week. It was calculated to contain 7.5 mg of iron, 1.2 mg thiamine, 70 gm protein, and 2400 calories. To hold the iron intake down to 7.5 mg necessitated the omission of only those foods that were extremely high in iron. The calcium, riboflavin and vitamin C were not calculated, as a large serving of citrus fruit every day insured sufficient vitamin C and a quart of milk insured sufficient calcium and riboflavin. The calculations

were made from the "Tables of Food Composition," prepared by the Bureau of Human Nutrition and Home Economics, and the National Research Council ('45) with the exception of the values for cooked meats taken from Bowes and Church ('46) and the value for the iron content of milk taken from Johnston ('44).

The basal diet was carefully controlled so that the intake for each week in the pre-beef period was exactly the same as that for the corresponding one in the beef period. The menu was planned on a weekly basis and was repeated so that it was the same on every succeeding corresponding day of the week. Paired cuts of meat were bought, 1 cooked immediately and 1 frozen for use on the corresponding day in the beef period. Both were cooked to the same internal temperature. All the fat was cut off and only lean meat weighed for the subjects. Canned goods were put up at 1 time in all-glass containers. All fresh and frozen vegetables and fruits were bought in 1 lot for the entire period. Distilled water was used for cooking, and drinking at meal time.

Foods allowed ad libitum were butter, sugar, coffee, a cola drink, tap water when away from the metabolic unit, and candy which was made from distilled water, sugar and other ingredients which were essentially iron free. Although these foods were fed ad libitum, a record was kept of the amount of each food eaten by each subject. The differences in the iron content of the diet in the pre-beef and beef periods due to variations in the amounts of these low iron foods were negligible. In a sense, the bread, rolls and biscuits were eaten ad libitum in that each subject decided the amount she desired during the 2 preliminary weeks and thereafter ate the same amount each week. An unenriched flour [1] was used so that the basal diet could be kept low in iron and at about the same level for all subjects.

[1] The flour was especially prepared for this project without enrichment by the Pillsbury Mills, Inc.

Care was taken to avoid any contamination during the preparation of the food. All cooking utensils were aluminum or glass and all knives were silver or stainless steel. Any places on the kitchen equipment which were susceptible to rust were coated with paint.

Weekly composites of the foods which all subjects ate in the same amounts were made in duplicate by different people. These were mixed in a Waring Blendor, treated with HCl, heated and stored in glass containers. The bread, biscuits, rolls, coffee, tap water and cola drinks were analyzed separately. For convenience the milk was analyzed separately although all subjects drank the same amount.

Carmine markers, for the separation of the stools, were given at the end of the 2-week adjustment period and at the end of each week thereafter. The weekly composites of the stools were digested with HCl and bottled.

The foods and stools were both wet-ashed with nitric and sulphuric acid, and the nitrosyl sulphuric acid hydrolyzed by boiling with water according to the method of Roberts, Beardsley and Taylor ('40). This method was deemed preferable to one employing perchloric acid as it was difficult to drive off all the perchloric acid in the samples of foods and stools taken after the addition of beef. The milk was dry-ashed in platinum dishes. The thiocyanate method of Stugart ('31) was used for the determination of iron. The colors were read in an Evelyn Photoelectric Colorimeter.

Four times, during the study, samples of venous blood were taken just before the noon meal and after a ¾ hour rest period to eliminate the effect of exercise. Serum iron was determined on 2 ml samples. The protein was precipitated by the method of Kitzes, Elvehjem and Schuette ('44) and iron determined by the o-phenanthroline method of Saywell and Cunningham ('37). All determinations were made in duplicate. Hemoglobin was determined by the method of Evelyn ('36) and red blood cell counts were made in duplicate on blood from each of 2 pipettes.

RESULTS

To find the absorption of iron from beef the following formula was applied:

$$\frac{\text{Iron in beef} - (\text{fecal iron on beef diet} - \text{fecal iron on basal diet})}{\text{Iron in beef}} \times 100$$

The iron absorptions for the 5 subjects were 32, 45, 58, 57, and 14% (table 1). Four of these women absorbed from $\frac{1}{3}$ to over $\frac{1}{2}$ the iron of the beef. Subject E, who was constipated much of the time, took mineral oil which caused a wide distribution of the carmine and made separation of feces between periods difficult. The stools of subjects A and B were normal while those of C and D were small and hard.

The retentions for subject E were especially high during the pre-beef period. This resulted in a paradoxical situation: according to the method of calculation used in this study, her percentage of absorption of beef iron was low, and yet on the beef diet she actually absorbed as much iron as subjects A and B.

Although this experiment was set up to offset the effect of iron "picked up" in the intestinal tract, absorptions may also be calculated on the diet as a whole if the amount of "picked-up" iron and iron secreted into the intestines is assumed to be negligible. In that case on the 7 mg daily intake, absorptions (food — feces) were 0.70, 0.32, 0.50, 0.69, and 1.40 mg or 10, 5, 7, 11 and 20% with a mean of 11% and on the 10.4 mg daily intake absorptions were 1.86, 1.85, 2.44, 2.61 and 1.84 mg or 18, 18, 23, 26 and 18% with a mean of 21%. Thus the percentage of iron absorbed (food — feces) was almost doubled after the addition of the beef.

The blood picture was one which showed all the subjects to be in good condition; they should have absorbed iron normally. Three of the subjects had serum iron levels of approximately 90 μg per 100 ml, one had a level of the 100 μg, and the other 121 μg. These are normal levels for the method employed. If need is a factor which increases iron absorption, subject E whose serum iron was 121 μg should have absorbed

TABLE 1

The retention of iron by 5 subjects from beef containing 3.4 mg per day.

	IRON					IRON					IRON				
	In food	In feces	Retained [1]	Retained from beef containing 3.4 mg Fe [2]		In food	In feces	Retained [1]	Retained from beef containing 3.4 mg Fe [2]		In food	In feces	Retained [1]	Retained from beef containing 3.4 mg Fe [2]	
	mg/day	*mg/day*	*mg/day*	*mg/day*	*%*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*%*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*%*
	Subject A					Subject B					Subject C				
Adjustment period:															
Week I	6.28					6.66					6.67				
Week II	6.68					6.85					6.89				
Basal period:															
Week III	6.87	6.42				6.85	5.98				7.02	6.26			
Week IV	6.94	5.65				7.09	7.94				7.10	6.52			
Week V	6.68	6.33				7.04	6.52				7.13	7.21			
Week VI	6.90	6.39				7.03	6.29				7.07	6.35			
Mean	6.90	6.20	0.70			7.00	6.68	0.32			7.08	6.58	0.50		
Beef period:															
Week VII	10.59	8.42				10.58	8.81				10.61	8.20			
Week VIII	10.37	7.58				10.52	7.98				10.54	7.85			
Week IX	10.41	9.23				10.45	8.75				10.59	7.61			
Week X	10.09	8.79				10.10	8.69				10.08	8.38			
Mean	10.36	8.50	1.86	1.10	32	10.41	8.56	1.85	1.52	45	10.45	8.01	2.44	1.97	58
	Subject D					Subject E									
Adjustment period:															
Week I	6.13					6.68									
Week II	6.22					6.63									
Basal period:															
Week III	6.44	5.16				7.00	5.43								
Week IV	6.59	6.07				7.13	4.60								
Week V	6.52	6.00				7.08	5.30								
Week VI	6.56	6.12				7.07	7.37								
Mean	6.53	5.84	0.69			7.07	5.67	1.40							
Beef period:															
Week VII	10.07	6.49				10.61	7.93								
Week VIII	10.03	8.15				10.54	8.23								
Week IX	9.97	6.22				10.59	10.56								
Week X	9.54	8.32				10.08	7.72								
Mean	9.90	7.29	2.61	1.95	57	10.45	8.61	1.84	.46	14					

[1] Derived from the 2 preceding columns.

[2] Calculated from the formula given in the text.

less than the others. She actually absorbed more than the others on the basal diet. Probably all the subjects had good stores of iron and in no case was the "need" great enough to raise absorptions above normal levels. The fluctuations in serum iron from week to week were normal. There was no indication of a drop during the 6 weeks on the 7 mg diet, nor

TABLE 2

The serum iron and hemoglobin values and red blood cell counts for the 5 subjects.

SUBJECT	A	B	C	D	E
Feb. 17 — Diet started		Iron in μg per 100 ml of serum			
Feb. 22 — Adjustment period	..	116	82	100	115
Mar. 15 — Pre-beef period	83	74	104	88	119
Mar. 29 — Pre-beef period	116	80	97	103	119
Apr. 12 — Beef period	76	43 [1]	90	110	129
Mean	92	90	93	100	121
Feb. 17 — Diet started		Hemoglobin in gm per 100 ml of blood			
Feb. 22 — Adjustment period	...	13.3	14.8	13.5	15.6
Mar. 8 — Pre-beef period [2]	13.7	13.6	14.0	14.0	14.9
Mar. 15 — Pre-beef period	13.0	12.8	13.3	14.0	14.9
Mar. 29 — Pre-beef period	13.2	13.7	13.9	13.3	15.4
Apr. 12 — Beef period	12.5	13.2	13.8	14.3	15.4
Mean	13.1	13.3	14.0	13.8	15.0
Feb. 17 — Diet started		Red blood cell counts in $\overline{M}$ per mm^3			
Feb. 22 — Adjustment period	...	4.37	4.67	4.40	4.45
Mar. 15 — Pre-beef period	4.28	4.61	4.05	4.75	5.13
Mar. 29 — Pre-beef period	4.28	4.93	5.52	4.81	4.92
Apr. 12 — Beef period	4.22	4.99	5.36	5.08	4.57
Mean	4.26	4.73	4.90	4.76	4.79

[1] Severe sore throat. This value was omitted in computing the mean.
[2] Capillary blood.

of a rise within 2 weeks after the beef was added. The hemoglobin concentrations for all 5 subjects were up to or above 13 gm (table 2), which is considered a satisfactory level for women. The red cell count for all the subjects except one was up to 4.5 $\overline{M}$ per mm^3 (table 2) which is considered an average count for women. The exception was a count of 4.26 $\overline{M}$ per mm^3 which is high enough to be satisfactory.

It may be noted that the diet which was calculated to contain 7.5 mg of iron was found by analysis to contain 7.0 mg (table 1). From this, one might conclude that when a diet is free from any sources of iron contamination and when the tables of food composition used in this study are employed, calculation yields values which are sufficiently accurate for many purposes.

DISCUSSION

Although this experiment was set up to determine the percentage of iron absorbed from beef, the results can be interpreted in another way: possibly the increase in absorption after the beef was added was not all due to iron present in the beef itself but to some effect of the beef on the food mixture so that there was an increase in the amount of iron absorbed from the other foods in the diet. Beef may be reducing in effect, changing the ferric iron to the ferrous form which is more readily absorbed. Tompsett ('40) has shown that at the pH of the stomach ferric iron is reduced by certain protein foods. Mirsky and Anson ('36) have shown that sulfhydryl groups in muscle proteins can reduce iron and, also, that tyrosine and tryptophane have some reducing properties although less than those of the sulfhydryl groups. Meat is a good source of pteroylglutamic acid (P.G.A.) and of Castle's extrinsic factor. It is possible that these substances and other functionally related ones aid in the absorption of iron. Mims, Swendseid and Bird ('47) found that sulfhydryl groups inactivated certain inhibitors of P.G.A. conjugase. Thus meat may not only supply P.G.A. but, also, compounds which allow it to be used.

If one assumes that all the increase in the iron retention after the addition of the beef came from the beef, the absorptions from beef found in this study would be high and the conditions of the experiment should be examined to see whether or not they are higher than could be expected for other women. These women were all 18 years of age. It is possible that they were still growing at a slow rate. The ex-

periment of Darby et al. ('47) with radioactive iron on children shows that growth increases the amount of iron absorbed. Leverton and Marsh ('42) believe that iron absorption is better on an adequate diet such as the one used in this study than on one which is deficient in one or more respects. In all 5 subjects the food-residue remained in the intestines for a rather long period of time. In most cases it remained 2½ to 3 days, 4 times it remained 4½ days, and for subject E it sometimes remained as long as 5½ days. Since the absorption of iron normally occurs in the duodenum and upper jejunum this circumstance probably did not increase absorption. There is little likelihood that men would absorb as much iron as did these young women because their needs are not as great.

The mean value of 11% found in this study for absorption from the basal diet, if "picked-up" iron and iron secreted into the intestinal tract are considered negligible, is similar to that of 12.5% found by Widdowson and McCance ('42) with women subjects, and to the top values found by Dubach et al. ('48) using radioactive iron. The sudden increase in absorption of iron after the addition of beef found in this study is similar to the sudden increase found by Leverton ('41) whose subjects were in fecal balance on 4–5 mg of iron and whose retentions jumped almost 3 mg upon the addition of 116 gm of beef. The behavior of Leverton's subjects was more extreme than in the case of these subjects. The former had been on a basal diet of lower iron content continued for a longer time than in the present case, previous to the addition of beef.

In planning this study it was decided that periods of at least 4 weeks should be used. Had periods of 2 weeks been used, the values for C and D would have been the same as for the 4 weeks, but the values for the 3 other subjects were definitely altered by the use of the longer period. A 5- or 6-week period might have increased the accuracy of the results, but it is questionable whether the added accuracy would have justified the added trouble and expense. In this experiment, as in all balance experiments, there was variation in fecal losses from week to week. There is no way of knowing how much of this

variation is due to changes in absorption and how much is due to errors in separation of the stools into periods. These "errors" are unavoidable as the food from each meal does not stay in a separate compartment as it travels through the gastrointestinal tract. The only way to minimize them is to use long metabolic periods.

SUMMARY

Five young women 18 years of age, in good health, with normal serum iron, hemoglobin and red cell counts were placed on a diet containing 7 mg of iron which was judged to be the lowest intake that would be adequate. For 2 weeks minor adjustments were made in the diet and the subjects were accustomed to a 7 mg level. During the next 8 weeks the adjusted basal diet was administered. During the last 4 of the 8 weeks 2 100-gm patties of ground beef ($\frac{2}{3}$ lean meat and $\frac{1}{3}$ fat) containing 3.4 mg of iron were added, 1 at breakfast and 1 at lunch. During the 4-week basal period and the 4-week beef period, weekly collections of feces were made and during the entire 10 weeks, weekly composites of the foods were preserved for analysis.

When the formula:

$$\frac{\text{Iron in beef} - (\text{fecal iron on beef diet} - \text{fecal iron on basal diet})}{\text{Iron in beef}} \times 100$$

was applied the absorptions from beef for 4 out of 5 subjects were 32, 45, 58 and 57%. This led to the conclusion that young women 18 years of age on a diet adequate in all respects absorb from about $\frac{1}{3}$ to more than $\frac{1}{2}$ of the iron of beef.

Another interpretation of the results is more likely the correct one: that the beef improved the absorption of the iron from the entire food mixture, rather than itself contributing all of the iron absorbed after it was added to the diet.

If the amount of iron added to the food residue as it passes through the gastrointestinal tract can be regarded as negligible, the mean absorption from the basal diet containing 7

mg of iron was 11% and was increased to 21% after the addition of beef to the 2 meals not already containing meat.

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THE EFFECT OF INANITION ON MAMMARY-GLAND DEVELOPMENT AND LACTATION [1]

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TWO FIGURES

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Several investigators (Marrian and Parkes, '29; Mason and Wolfe, '30; Moore and Samuels, '31; Pomerantz and Mulinos, '39; Werner, '39, and Mulinos and Pomerantz, '40) have presented data which suggest that inanition causes a pituitary insufficiency. Mulinos and Pomerantz ('40) have designated the condition produced by inanition as pseudo-hypophysectomy because of the similarity of the effects of inanition and of hypophysectomy on various endocrine organs. Therefore, because inanition affects the endocrine organs, it is not surprising that it should retard the development of the mammary gland. This retarding effect has been noted by many workers (Astwood, Geschickter and Rausch, '37; Nathanson, Shaw and Franseen, '39; Trentin and Turner, '41; Huseby and Ball, '45). All these studies on mammary growth have involved the use of non-pregnant animals and, although Trentin and Turner ('41) have shown that the effectiveness of estrogen in inducing mammary growth parallels food intake, no data are available as to whether the estrogen produced during pregnancy would overcome the effect of inanition.

[1] This research was supported by an appropriation from Bankhead-Jones special research funds (Bankhead-Jones Act of June 29, 1935).

The present experiment was undertaken, therefore, to determine the effect of inanition on mammary growth when feed restriction was imposed from weaning through pregnancy and to correlate mammary growth with subsequent lactation.

PROCEDURE

One hundred and twenty female white rats weaned at 25 to 26 days of age were divided into 2 groups of 60 each, with equal numbers of litter mates in both groups. They were housed 10 rats to a cage.

TABLE 1

Composition of diets.

	STOCK DIET (CONTROL)	MODIFIED STOCK DIET (RESTRICTED)
	lb.	*lb.*
Corn meal	69.5	52.5
Linseed meal	14.0	19.0
Meat scrap	9.0	12.6
Casein	4.0	10.0
Alfalfa-leaf meal	2.0	2.8
Poultry bone meal	2.0	2.8
Salt	0.5	0.7

Group 1 (controls) were fed a stock diet in such amounts that minimal quantities of feed remained in the feed cups each morning. They can be considered to have been fed ad libitum. Group 2 animals (restricted intake) were fed the same diet as that fed to group 1, but the total amount of the diet fed was only 70% of that fed to group 1. The ingredients were so adjusted that, although the energy intake of these rats was reduced by 30%, they received the same amounts of protein and other food nutrients as the animals in group 1. The composition of the diets is shown in table 1. Lettuce and raw carrots were also fed once weekly. This stock diet supplemented with lettuce and carrots permits good growth, reproduction, and lactation in our colony.

All rats were mated at 100 days of age. At this time the number of females was reduced to 5 per cage, and the males were left with them for 14 days and alternated between cages at the end of 7 days. No further opportunity for mating was afforded.

At parturition, half of the rats in each lot were sacrificed. The 3 posterior mammary glands on the left side were dissected out, after removing and stretching an adequate area of hide together with the glands. The thyroid, adrenal, and pituitary glands were also obtained. Weights of the glands were determined. It was not possible to determine comparative degrees of mammary development by histological examination in these rats and while it was recognized that varying amounts of other tissue, particularly fat, between the 2 groups might seriously interfere with the validity of deductions based on weight data, this was the only quantitative measure at our disposal.

The remaining rats of both groups which bore young were allowed to suckle a litter of 12 for 21 days. Litter weights were obtained every other day during this period. The gain in weight of the litter was used as a measure of the lactation ability of the mother. All mother rats, whether or not they had been restricted in food intake prior to parturition, were placed on the stock diet during the nursing period and fed ad libitum. At the end of the nursing period the mother rats were killed and the weights of the mammary glands determined, as was done with the rats that were sacrificed at parturition.

Those rats that did not become pregnant were disposed of as soon as it was certain that they were not carrying young. They were killed at an age, therefore, which on the average was only slightly greater than that of the rats sacrificed at parturition. Mammary, thyroid, adrenal, and pituitary glands were obtained from these animals. In these non-pregnant rats, microscopic examination was the only means by which the comparative mammary development of rats on the control and restricted diets could be determined, since the limits of

the area of glandular development were so poorly defined that an accurate dissection was impossible.

RESULTS

Mammary development

Data on mammary-gland weights are shown in table 2. The average weight of the dissected mammary glands from rats that were killed at parturition was 3.34 gm for those on the control diet and 1.87 gm for those on the restricted diet. Glands from the control group were thus 80% heavier than those from the restricted group. The body weights of these 2 groups

TABLE 2

The effect of reduced feed intake on mammary-gland development.

	NUMBER OF RATS	AVERAGE WEIGHT OF MAMMARY GLAND	AVERAGE BODY WEIGHT AT AUTOPSY
		gm	*gm*
Group 1 (control)[1]	24	3.34	230.8
Group 2 (restricted)[1]	19	1.87	188.3
Group 1 (control)[2]	23	3.55	238.5
Group 2 (restricted to parturition)[2]	18	3.51	220.4

[1] Size of glands determined on day of parturition.
[2] Size of glands determined following a 21-day lactation period.

at autopsy averaged 230.8 and 188.3 gm, respectively, a difference of only 22.6%. While these values do not represent differences in the amount of secreting tissue alone, it is our opinion that they do represent comparative development. The exact magnitude of development, however, is unknown. That the values do not represent apparent differences which might in reality be an accompaniment of reduced body size or deposition of fat, or both, would appear to be indicated by the marked difference in gland weight (80.0%) as compared with the relatively small difference in body size (22.6%) of the 2 groups. The differences noted in mammary-gland weight were undoubtedly due in part to the relative amounts of fat de-

posited in the area of the gland; but even if one assumes that the amount of fat deposited in this area is less than in other parts of the body when animals are on a restricted dietary intake, the disproportion between gland size and body size was of sufficient magnitude as to make it highly improbable that a difference in fat deposition would by itself account for the effects noted. Variation in the amount of secretion present in the tissues, at the time they were removed and weighed, was kept to a minimum by leaving the litters with the mothers until just before autopsy. The 2 illustrations (figs. 1 and 2)

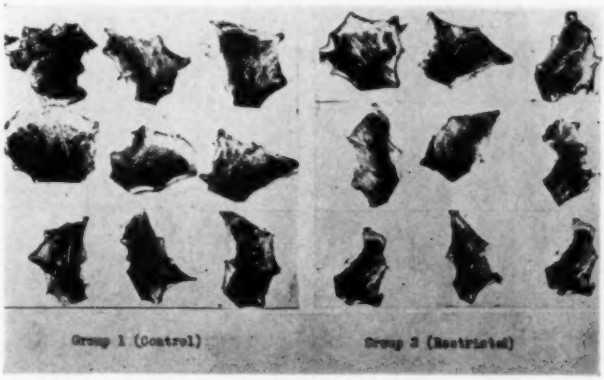

Fig. 1 Rat mammary glands obtained at parturition.

also indicate that there was a real difference in the mammary development of the 2 groups.

The individual glands shown in figure 1 were obtained from rats which bore young and were sacrificed at parturition. The glands were stretched on sheet cork, fixed in Bouin's fixative, washed and taken into 70% alcohol. The abdominal gland was separated as accurately as possible from the 2 smaller, more compact inguinal glands and photographs of the former were made with transmitted light. The glands shown in figure 1 are representative of the type of development observed. The upper 6 glands from the control rats are most

representative of the development seen in this group, while the lower 6 glands from the restricted rats represent the type of development observed in 80% of the rats on restricted intake. While differences in the density of glandular tissue are not marked, the extent of development is obviously greater in the control rats.

The glands shown in figure 2 were obtained from non-pregnant rats which were killed at approximately the same age as those whose glands are shown in figure 1. The gland on

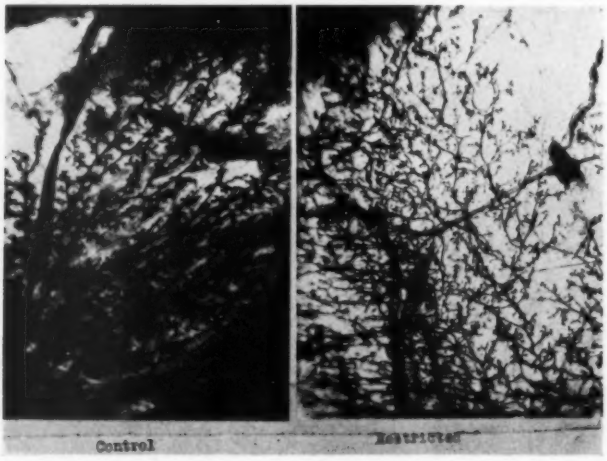

Fig. 2 Mammary glands from non-pregnant rats.

the right is one of the most highly developed glands observed in the non-pregnant restricted animals, while that on the left is representative of the average development of the control rats.

These results would therefore indicate that inanition reduces the development of mammary tissue in rats which become pregnant as well as in non-pregnant animals (Huseby and Ball, '45) or animals (Trentin and Turner, '41) in which mammary growth has been stimulated by estrogenic hormones. The retardation of development at parturition in our

animals would appear to be quantitative rather than qualitative. The glands at this time appeared mature in every respect in both the restricted and the control rats and, as will be shown, were capable of reasonably adequate lactation in both lots.

Whether or not this reduction in mammary growth was brought about indirectly through the effects of inanition on the endocrine system, as might be suggested from the work of Mulinos and Pomerantz ('40), is not clear from our data. The average weights of certain endocrine glands from restricted and control rats are given in table 3.

TABLE 3

Comparison of endocrine glands of rats on full feed and on restricted diet.

ANIMAL GROUP	WEIGHT OF		
	Pituitary	Adrenal	Thyroid
	mg	*mg*	*mg*
Killed at parturition			
Rats on full diet	11.8	51.7	23.0
Rats on restricted diet	9.9	45.2	17.6
Non-pregnant animals			
Rats on full diet	13.2 [1]	45.5 [1]	27.0
Rats on restricted diet	8.7	34.2	22.7

[1] The difference in weight was greater than might be expected from the difference in body weight in these 2 instances only.

The weights of the pituitary, adrenal, and thyroid glands were smaller throughout in the restricted rats but, except in the case of the pituitary and adrenal glands of the non-pregnant rats, this reduction in size was proportional to the reduction in body size. There may therefore have been some hormone deficiency in the non-pregnant (restricted) rats or in all the restricted rats up to the time when pregnancy occurred, if it is permissible to draw such a conclusion by using this disproportionality between gland weights and body weight as a basis. That this may have been so is also suggested by the slightly delayed time of vaginal opening and fewer number of pregnancies observed in the restricted rats. Ball,

Barnes and Visscher ('47) have noted a similar reduction of pregnancies in underfed mice. This deficiency, if present, appeared to disappear during pregnancy as judged by the fact that the endocrine gland weights were proportional to body weight at parturition. We were unable to demonstrate a difference in prolactin content of the pituitary glands which were removed at parturition. It would seem, therefore, that a hormone deficiency was not a major factor in the reduced mammary development observed at parturition.

When rats, which were underfed until parturition, were fed ad libitum during a 21-day lactation period, they gained in body weight rapidly until at the end of lactation they had attained body weights nearly equal to those of rats on full feed from weaning. A similar rapid increase in body weight was noted by Ball et al. ('47) when feed consumption was increased in their previously underfed mice. The average weights of the mammary glands had also become equal in the 2 groups. These data are also shown in table 2. During this period it would appear that a much more rapid growth of mammary tissue occurred than was the case with the other tissues. There was no apparent gain in the mammary tissue in group 1 during this period.

Lactation

In spite of the fact that less mammary tissue was present at parturition in the restricted rats, these mothers raised superior litters. During this time they were fed ad libitum in order to eliminate the effect of reduced feed intake on lactation. Their feed consumption, as compared with that of the controls, must actually have been greater in relation to body size, since they gained in body weight. The gain per litter, the average weight of the young, and the survival of the young at the fourteenth and twenty-first days of lactation were greater in every instance in the case of mothers which had been subjected to reduced feed intake. These data are presented in table 4.

These animals, then, not only increased the amount of secreting tissue during this period, as shown in table 2, but they also secreted milk at a better rate than rats whose mammary glands had attained mature size at parturition. Possibly, the explanation for this difference in lactation performance is that regression of the functional activity of the mammary tissue may have set in relatively early during lactation in those glands that were fully developed at parturition (glands from rats of group 1). This regression in function apparently did not occur, or it was reduced in extent in the glands of the previously restricted rats (group 2) which were actively growing during the lactation period and were therefore presumably in a more viable condition. The similarity of the

TABLE 4

The effect of inanition on lactation of rats during growth and pregnancy.

	AVERAGE WEIGHT PER LITTER AT START	AT 14 DAYS			AT 21 DAYS		
		Total gain per litter	Average weight of young	Surviving	Total gain per litter	Average weight of young	Surviving
	gm	*gm*	*gm*	%	*gm*	*gm*	%
Group 1 (control)	68.4	139.1	18.0	96.4	163.8	23.9	76.5
Group 2 (restricted)	63.6	154.5	18.4	98.7	198.3	25.3	86.0

effects of inanition on lactation obtained in this study with those obtained on reproduction by Ball, Barnes and Visscher ('47) is rather striking.

The degree of inanition which was imposed on these animals was not particularly severe. Reduction in body size occurred and the reproductive functions were apparently somewhat subnormal. The animals, however, appeared healthy at all times; there was no mortality, and they rapidly attained mature body size during lactation when adequate feed was available. In animals which are to be used for milk production, it may therefore be desirable to regulate food intake so that only moderate body size and mammary-gland development are attained at the time of first parturition, in order to attain the best results in the subsequent lactation periods.

SUMMARY

Inanition in rats from weaning through pregnancy reduces growth of the mammary gland to a greater extent than might be expected from the reduction in body size which results from reduced feed intake.

When adequate feed was supplied during lactation to rats that had been on the restricted diet, they increased in body size rapidly, the mammary gland also grew rapidly, and their lactation performance was superior to that of rats that were on adequate feed intake throughout the whole of the experimental period.

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CYSTINE AND METHIONINE METABOLISM BY CHICKS RECEIVING RAW OR AUTOCLAVED SOYBEAN OIL MEAL[1]

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Autoclaving soybean oil meal affects the nutritive value in 2 ways. Moderate autoclaving (such as at 100°C.–120°C. for 30 minutes or equivalent) has been shown to increase the nutritive value of raw soybean oil meal for growing chicks (Hayward et al., '37; Almquist et al., '42; Bird and Burkhardt, '43; Parsons, '43; Evans and McGinnis, '46), whereas a more drastic autoclaving, such as at 130°C. for 60 minutes, has been shown to decrease the nutritive value of the moderately autoclaved meal (Bird and Burkhardt, '43; Parsons, '43; Evans and McGinnis, '46; Clandinin et al., '47). The lowering of the nutritive value of proteins caused by heat-treatment has been recognized for several years (McCollum et al., '39) but only recently has it been shown that the nutritive value of soybean proteins is also injured by heat-treatment. The influence of heating on the soybean proteins is generally believed to be related to the methionine availability and metabolism (Melnick et al., '46).

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The present investigation was a study of the biochemical changes occurring in soybean oil meal proteins as a result of autoclaving and of the effects of these changes on cystine and methionine metabolism by the chick. Earlier work from this laboratory (Evans and McGinnis, '46) indicated that autoclaving selectively affected the availability of methionine, moderate autoclaving (100°–120°C. for 30 minutes) increasing methionine retention, and drastic autoclaving (130°C. for 30 or 60 minutes) decreasing it. This early work was not conclusive since gelatin and dried brewers' yeast furnished 40% of the dietary protein. In the present study soybean oil meal was the only source of dietary protein. The earlier work indicated that the cystine availability was also affected. Since no supplemental cystine was fed, it was not possible to determine the extent to which cystine could replace methionine as a supplement to the raw or over-autoclaved soybean oil meals.

EXPERIMENTAL

Groups of 20 New Hampshire chicks were fed a diet containing: cerelose 43.9 gm, soybean oil meal 48.0 gm, minerals [3] 5.0 gm, soybean oil 2.5 gm, fortified fish oil 0.5 gm, liver paste 0.1 gm, choline chloride 200 mg, tocopherol concentrate 10 mg, p-aminobenzoic acid 10 mg, nicotinic acid 3 mg, 2-methyl-1,4-naphthoquinone 1 mg, riboflavin 1 mg, calcium pantothenate 1 mg, thiamine 0.5 mg, pyridoxine 0.5 mg, pteroylglutamic acid 0.05 mg, and biotin 0.01 mg. The soybean oil meals represented 3 different types: (1) raw meal, (2) raw meal autoclaved for 30 minutes at 100°C. (this might be better referred to as a steaming treatment since little pressure was used), and (3) raw meal autoclaved for 60 minutes at 130°C. Groups of chicks fed the above soybean oil meals also received supplements of 0.5% L-cystine, 0.5% DL-methionine, and 0.5% cystine + 0.5% methionine. Chicks fed the more drastically auto-

[3] The composition of the mineral mixture was: oyster shell, 1570 gm; $CaHPO_4 \cdot 2H_2O$, 1740 gm; K_2HPO_4, 840 gm; NaCl, 600 gm; $MgSO_4 \cdot 7H_2O$, 500 gm; $Fe_2(SO_4)_3 \cdot XH_2O$, 55 gm; $MnSO_4 \cdot 4H_2O$, 29 gm; KI, 3.3 gm; $CaSO_4 \cdot 5H_2O$, 1.5 gm; $ZnCl_2$, 1.0 gm; and $CoCl_2 \cdot 6H_2O$, 0.2 gm.

claved meal also received supplements of 0.5% L-lysine and a combination of 0.5% cystine + 0.5% methionine + 0.5% lysine.

Sulfur balance studies were conducted with each group of chicks for 3 days during the third week of the experiment. All of the chicks in a group were considered as a single unit for the balance study. Cystine, methionine, and sulfate sulfur intakes were determined from the dietary feed intake and composition. Droppings were collected on glass plates and rapidly air-dried. Cystine, methionine, and sulfate sulfur were determined on the feed and the droppings by the differential oxidation procedure (Evans, '45). Excretion and retention of these were calculated.

Methionine, cystine, and lysine determinations were also made on acid and enzyme digests of the 3 soybean oil meals by microbiological procedures. Acid hydrolysates were prepared by autoclaving 1-gm samples in sealed tubes with 10 ml of 10% hydrochloric acid, diluting, neutralizing, and making to a suitable volume. *In vitro* trypsin-erepsin digests were prepared as described by Evans ('46). Lysine was assayed microbiologically, using *Streptococcus faecalis R* (Baumgarten et al., '46), and methionine and cystine were assayed with *Lactobacillus arabinosus* 17-5 (Riesen et al., '46). Amino nitrogen was determined by the method of Van Slyke ('13).

RESULTS

Cystine and methionine balance data for the chicks fed the unsupplemented soybean oil meals are presented in table 1. Over 50% of the ingested cystine was excreted in the droppings unchanged. The percentage of the ingested cystine sulfur which was not excreted unchanged is reported as cystine sulfur retained plus that excreted as sulfate. This value was obtained by subtracting the cystine sulfur excreted unchanged from the cystine sulfur ingested. It was not possible to determine how much of the "retained plus excreted as sulfate" cystine sulfur was retained for building body proteins and how much was oxidized to sulfate and excreted

as such. Similar calculations were made with the methionine data.

Data are also presented in table 1 for the cystine plus methionine sulfur excreted unchanged, cystine plus methionine sulfur oxidized to sulfate and excreted, and cystine plus methi-

TABLE 1

Cystine and methionine balance of growing chicks fed raw and autoclaved soybean oil meals.

	NOT AUTOCLAVED	AUTOCLAVED AT 100°C. FOR 30 MIN.	AUTOCLAVED AT 130°C. FOR 60 MIN.	AUTOCLAVED AT 130°C. FOR 60 MIN. + LYSINE
Cystine sulfur				
Intake (gm)	0.80	2.03	1.54	1.35
Excreted as cystine (gm)	0.62	1.07	1.07	0.91
Excreted as cystine (%)[1]	78	53	70	67
Retained + excreted as sulfate (%)[1,2]	22	47	30	33
Methionine sulfur				
Intake (gm)	0.36	0.92	0.70	0.62
Excreted as methionine (gm)	0.15	0.15	0.26	0.32
Excreted as methionine (%)	42	16	37	52
Retained + excreted as sulfate (%)[2]	58	84	63	48
Cystine + methionine sulfur				
Intake (gm)	1.16	2.94	2.23	1.96
Excreted as cystine and methionine (gm)	0.78	1.22	1.33	1.23
Excreted as cystine and methionine (%)	67	42	60	63
Retained + excreted as sulfate (%)[2]	33	58	40	37
Oxidized to sulfate (gm)	0.11	0.24	0.13	0.06
Oxidized to sulfate (%)	10	8	6	3
Retained (%)	23	50	34	34
Protein efficiency (chicks)[3]	1.4	2.1	1.5	1.5

[1] Per cent of total cystine intake.

[2] This value represents the percentage of total cystine or/and methionine in the soybean oil meal which was not excreted unchanged. Part of this was retained for building body proteins and part was oxidized to sulfate and excreted as such. See text.

[3] Grams gain in weight per gram of protein consumed.

onine sulfur retained. The latter value was determined by subtracting the sulfur excreted from the total sulfur intake. It was not possible to determine the proportions of the sulfur retention representing the sulfur in cystine and methionine, respectively, because of the inability to distinguish between the sulfate formed from cystine and that from methionine.

Moderate autoclaving, or steaming (100°C. for 30 min.), increased both the retention of cystine plus methionine sulfur by growing chicks and the protein efficiency, the latter being determined by the gain per gram of protein consumed (table 1). Both cystine and methionine were retained or oxidized to sulfate before excretion to a greater extent when the soybean oil meal was steamed.

The methionine plus cystine sulfur of the drastically autoclaved soybean oil meal (130°C. for 60 minutes) was retained by growing chicks to a lesser extent than that of the steamed meal, but to a slightly greater extent than that of the raw meal.

The addition of 0.5% lysine to the over-autoclaved soybean oil meal did not improve cystine plus methionine retention or protein efficiency.

Data concerning the metabolism of supplemental cystine and methionine are presented in table 2. For calculating these data it was assumed that the same percentages of the cystine and methionine contained in the soybean oil meal were excreted unchanged, retained,.and oxidized to sulfate in the same manner as when no supplement was added. Any increases in these values were considered to come from the supplement.

About 30% of the supplementary cystine was retained by the chicks fed the raw and drastically autoclaved soybean oil meals when 0.5% L-cystine was added to the diet. Less was retained by the chicks fed the steamed meal. About 50% of the supplemental cystine was oxidized to sulfate and excreted as such, and 10 to 30% was excreted unchanged. Of a 0.5% methionine supplement, more than 50% was retained and 92% retained and metabolized by the chicks fed the raw soybean

TABLE 2

The metabolism of supplementary cystine and methionine by growing chicks fed raw and autoclaved soybean oil meals.

	NOT AUTOCLAVED	AUTOCLAVED AT 100°C. FOR 30 MIN.	AUTOCLAVED AT 130°C. FOR 60 MIN.	AUTOCLAVED AT 130°C. FOR 60 MIN. + LYSINE
	% [1]	% [1]	% [1]	% [1]
Supplement — 0.5% cystine				
Excreted as cystine	10	27	20	
Excreted as methionine	3	0	5	
Retained + excreted as sulfate	87	73	75	
Excreted as sulfate	56	55	45	
Retained	31	18	30	
Increased protein efficiency [2]	7	10	0	
Supplement — 0.5% methionine				
Excreted as methionine	5	3	14	
Excreted as cystine	1	5	9	
Retained + excreted as sulfate	94	92	77	
Excreted as sulfate	38	58	57	
Retained	56	34	20	
Increased protein efficiency [2]	29	5	0	
Supplement — 0.5% cystine + 0.5% methionine				
Cystine excreted as cystine	23	24	1	17
Cystine retained + excreted as sulfate	77	76	99	83
Methionine excreted as methionine	2	3	26	28
Methionine retained + excreted as sulfate	98	97	74	72
Cystine + methionine:				
Excreted unoxidized	14	15	13	22
Retained + excreted as sulfate	86	85	87	78
Excreted as sulfate	57	52	41	37
Retained	29	33	46	41
Increased protein efficiency [2]	14	5	7	33

[1] Per cent of supplemental cystine, methionine, or cystine + methionine consumed.

[2] The increased protein efficiency represents the percentage increase in protein efficiency resulting from the supplement or supplements added. For example, the protein efficiency for the raw meal was 1.4 and for the raw meal supplemented with 0.5% DL-methionine 1.8, an increase of $\frac{0.4}{1.4} \times 100 = 29\%$.

oil meal. This is in contrast to the 20% retained and 77% retained and metabolized by the chicks receiving the drastically autoclaved meal. Thirty-eight to 58% of the supplementary methionine was converted to sulfate and then excreted (metabolized).

Chicks fed a diet containing the drastically autoclaved soybean oil meal supplemented with both cystine and methionine retained and metabolized practically all of the supplemental

TABLE 3

The influence of autoclaving soybean oil meal on the liberation of methionine, cystine and lysine by acid and by in vitro trypsin and erepsin digestion.

	METHOD OF HYDROLYSIS	NOT AUTOCLAVED		AUTOCLAVED 30 MIN. AT 100°C.		AUTOCLAVED 60 MIN. AT 130°C.	
		In soybean oil meal	Enzyme/ Acid	In soybean oil meal	Enzyme/ Acid	In soybean oil meal	Enzyme/ Acid
		%	%	%	%	%	%
Amino nitrogen	Acid	5.50		5.48		5.30	
	Enzyme	1.65	30	2.28	42	1.77	33
Methionine	Acid	0.71		0.71		0.75	
	Enzyme	0.35	49	0.50	70	0.40	53
Cystine	Acid	0.95		0.98		0.58	
Lysine	Acid	3.0		3.1		2.2	
	Enzyme	0.9	30	1.1	35	0.1	5

cystine but only 74% of the supplemental methionine compared to a retention and metabolism of 76% of the supplemental cystine and 98% of the supplemental methionine by the chicks fed the other 2 meals. The addition of 0.5% L-lysine did not improve cystine or methionine utilization or retention.

The results of the amino acid determinations on acid and enzyme digests of the soybean oil meals are presented in table 3. Steaming did not influence the methionine, cystine, or lysine contents. It did, however, increase the amounts liberated by enzyme digestion. More drastic autoclaving did not change the methionine content of the meal but destroyed 40% of the cystine and 30% of the lysine as determined by bacterio-

logical assay. There was a decreased liberation of methionine by enzymic digestion, which paralleled the decreased liberation of amino groups. Very little of the lysine was liberated by enzymic digestion. Cystine determinations on the enzymic digests could not be made because cystine peptides exert a greater stimulating action on *L. arabinosus* than does free cystine.

DISCUSSION

It is generally believed that raw soybean oil meal is deficient only in methionine for the growing chick. The data presented in this paper indicate that such is not the case but that raw soybean oil meal is deficient in essential amino acids other than methionine. Methionine additions to the raw soybean oil meal did not increase growth and protein efficiency (body weight gain of chicks per gram of protein consumed) to the extent that mild autoclaving did (McGinnis and Evans, '47). The data of Almquist et al. ('42) and Evans and McGinnis ('46) indicating only a methionine deficiency in raw soybean oil meal were obtained with diets containing considerable gelatin. Gelatin contains only low levels of cystine and methionine, but is a fair source of some of the other amino acids (Mitchell and Block, '46). Since methionine is the most limiting amino acid in soybean oil meal (Mitchell and Block, '46), methionine additions to the diet improved growth and protein utilization, but did not give maximum growth.

The present data can be explained by the poor digestion by the chick of the proteins of raw soybean oil meal (Evans, McGinnis and St. John, '47). A trypsin inhibitor has been shown to be present in raw soybean oil meal (Ham and Sandstedt, '44; Bowman, '44). When this inhibitor was added to diets containing autoclaved soybean oil meal, fish and meat meals, or casein, the protein nutritive value was decreased for chicks (Ham et al., '45) and rats (Klose et al., '46). This trypsin inhibitor is destroyed by heat.

Drastic autoclaving of soybean oil meal decreases the protein nutritive value by decreasing the availability of the lysine, and the methionine or cystine or both, since the nutritive value

can be restored to optimal by supplementing with 0.34% DL-methionine and 0.65% L-lysine monohydrochloride (Clandinin et al., '47), or with 0.5% DL-methionine, 0.5% L-cystine, and 0.5% L-lysine (McGinnis and Evans, '47). Parsons ('43) earlier observed that drastic autoclaving (123°C. for 90 minutes) decreased the protein nutritive value of soybeans for rats and that supplementation with 0.2% D-lysine partially corrected this. The lysine of soybean oil meal was partially destroyed by the drastic autoclaving. Of the remaining lysine, part was converted to a form which was not liberated from the proteins by enzymic digestion as shown by Riesen et al. ('47) and by the present studies.

Drastic autoclaving of soybean oil meal destroyed part of the cystine, according to the microbiological assays, but not according to the differential oxidation procedure. This destruction may have been by racemization or by conversion to some readily oxidized, biologically inactive compound. The destruction was of sufficient magnitude to explain the increased excretion (biologically active or inactive) and the decreased retention and metabolism of cystine by the chick.

No mechanism for the selective inactivation of methionine by drastic autoclaving has been advanced. No destruction of methionine, as shown by microbiological assay on the acid-digested meal, was observed by Riesen et al. ('47) or in the present study. Russell et al. ('46) observed that much of the methionine in certain legumes was not available to the growing rat, but could offer no reason for this.

The microbiological data in table 3 show that drastic autoclaving of the soybean oil meal decreased the percentage of methionine which was liberated by *in vitro* trypsin and erepsin digestion. Evans, McGinnis and St. John ('47) observed a "selective concentration" of methionine in the protein fraction of drastically autoclaved soybean oil meal not digested by the chick. Their data show that 6.7% of the methionine sulfur of the steamed meal was in the undigested protein fraction while 25.6% of that of the drastically autoclaved meal was in this fraction, an increase of 3.8 times. The same

data show that 11.5% of the protein of the steamed soybean oil meal was not digested, compared with 16.1% of the protein of the drastically autoclaved meal, or an increase of 1.6 times.

The data of table 2 indicate that metabolism of the supplemental methionine by the chick was interfered with when the diet contained the drastically autoclaved soybean oil meal. Retention of supplementary methionine by the chick was reduced from 33% on the steamed soybean oil meal to 20% on the drastically autoclaved meal. Chicks fed the raw meal retained 54% of the supplemental methionine. It appears that autoclaving soybean oil meal at 130°C. for 60 minutes may have caused the formation in the meal of some substance or substances which interfered with the normal absorption or metabolism, or both, of methionine. Part of the methionine, both that present in the soybean oil meal and that in the supplement, was apparently "tied up" in such a manner that it could not be utilized for tissue building. Considerable methionine was excreted unchanged (possibly unabsorbed) and part was oxidized to sulfate and excreted.

SUMMARY

Mild autoclaving, or steaming (100°C. for 30 minutes), of a raw soybean oil meal increased the percentage of the cystine and methionine present in the meal that was retained or metabolized by the growing chick. Steaming apparently increased the digestibility of the soybean oil meal proteins, probably by a destruction of the trypsin inhibitor.

More drastic autoclaving (130°C. for 60 minutes) of a raw soybean oil meal reduced the percentage of the cystine and methionine present in the meal that was retained or metabolized as compared to the steamed meal.

Retention of supplemental L-cystine by the chicks fed the drastically autoclaved soybean oil meal was the same as that for the chicks fed the raw meal. Retention of the supplemental DL-methionine was, however, decreased from 56% of the intake for chicks fed the raw meal to 20% for those fed the drastically autoclaved meal, and supplemental methionine excretion

was increased from 3% to 26%. The formation, by drastic autoclaving, of a substance in the soybean oil meal which interfered with normal digestion and metabolism of methionine is indicated. The addition of supplemental L-lysine to the diet did not affect the cystine and methionine metabolism.

Autoclaving did not influence the methionine content of soybean oil meal as determined microbiologically. Drastic autoclaving destroyed 40% of the cystine as shown by a similar assay.

The liberation of methionine from soybean oil meal by *in vitro* digestion with trypsin and erepsin was increased by steaming but again decreased by drastic autoclaving.

The drastic autoclaving procedure reduced the nutritive value of soybean oil meal by (1) a partial destruction of cystine and lysine, (2) a decreased digestibility of the lysine not destroyed, and (3) a decreased absorption and utilization of the methionine, probably caused by the formation of a substance which interfered with normal digestion and metabolism of methionine.

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THE EROSIVE ACTION OF VARIOUS FRUIT JUICES ON THE LOWER MOLAR TEETH OF THE ALBINO RAT [1]

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The erosive action of a dilute solution of hydrochloric acid, grapefruit juice and cranberry juice cocktail on the molar teeth of the albino rat (McClure, '43) suggests that similar effects might be expected from any other acid-containing beverage. From the dietetic point of view this is a matter of considerable significance because of the acidic nature of many fruit juices that have an extensive commercial distribution, and inferentially have become fairly common articles of diet. The present study was undertaken to obtain comparative experimental data on the action of a number of these juices on the teeth.

The experiment consisted of 2 parts. In the first part, the juices served as the sole source of drinking fluid for the test animals; in the second, the juices were administered 3 times a day in measured amounts and constituted only a portion of the fluid intake.

PROCEDURE

Part 1. Fruit juices constituting the entire fluid intake

Weanling rats were selected from the colony and placed in individual cages. Thenceforth throughout the duration of the

[1] The expense of this investigation was defrayed in large part by a grant-in-aid from the Sugar Research Foundation.

experiment extending over 100 days, they were fed a commercial dog food [2] exclusively which they were allowed to eat *ad libitum*. The sole source of fluid of each test animal during this time was one of the following juices: apple, grape, orange, tomato, unsweetened grapefruit to which 10 gm sugar per 100 ml was added, pineapple and prune. Sugar was added to the grapefruit juice because the animals would not drink it unsweetened. These juices, all of which were canned, were purchased at random from the shelves of a neighborhood grocery store with no consideration given to the trade name. The juices that contained pulp were centrifuged and only the supernatant fluid free from solid material was used. The control animals were allowed to drink water *ad libitum*.

The daily consumption of the various juices by the weanling rats increased rapidly day by day and amounted to 35 ml within less than 2 weeks. Thereafter the daily intake was restricted to this amount. This was somewhat in excess of the daily water intake of the controls which amounted to an average of 26 ml. In the course of the experiment each test animal drank a total of 3.5 liters of fruit juice. It was considered advisable to limit the intake of the juices to a definite amount because the rat is not temperate in its drinking habits when given a fluid that appeals to its taste. Unless restricted, the daily consumption of a delectable fluid will exceed by several times the normal intake of water.

At the conclusion of the experiment the animals were decapitated. After boiling the heads in water for 30 minutes the teeth were removed, dried and weighed. They were then studied under the microscope and the degree of erosion evaluated by following in general the criteria of Restarski, Gortner and McCay ('45). High polish of lingual enamel, slight etching, mild destruction, moderate destruction and severe destruction were graded, respectively, 1, 2, 3, 4 and 5. The teeth were then redried and weighed and the percentage enamel determined by the procedure of Manly and Hodge ('39). This method does not yield absolutely accurate results, as a few of

[2] Purina dog chow.

the enamel particles cling to the dentine in the separation of the 2 fractions. However, it suffices for a relatively good degree of quantitative approximation for a comparative evaluation of erosion.

RESULTS

The data of the experiment on the lower molars are presented in table 1. Similar data were obtained on the upper as on the lower molars, but these are omitted from the table for the reason that the uppers showed very little erosion except for those of the test group on grapefruit juice. In no

TABLE 1

Effects of various juices on the lower molar teeth of the white rat when consumed as the sole drinking fluid for 100 days.

FLUID CONSUMED	WEIGHT OF LOWER MOLARS	LOSS IN WEIGHT	DEGREE OF EROSION [1]	WEIGHT OF ENAMEL	LOSS IN WEIGHT OF ENAMEL	TITRATABLE ACID	pH	NUMBER OF ANIMALS
	mg	%		*mg*	%	%		
Water	69.3	..	0	15.2	...	...	..	36
Tomato juice	64.5	6.9	1	12.7	16.4	0.37	4.2	9
Prune juice	65.2	5.9	1	12.2	19.7	0.54	3.6	7
Orange juice	63.3	8.6	3	11.8	22.4	1.08	3.6	8
Pineapple juice	64.1	7.5	2	11.6	23.7	0.78	3.4	12
Apple juice	62.4	10.0	4	11.4	25.0	0.54	3.5	10
Grape juice	61.5	11.3	4	10.7	30.0	0.98	3.1	11
Grapefruit juice (sweetened)	58.4	15.7	5	9.1	40.0	1.90	3.1	13

[1] Microscopic evaluation.

instance was the average weight of the enamel of the upper molars of the test animals less than that of the controls.

The values in the table, including the microscopic evaluation, are averages of all 6 lower molars. The degree of erosion was not always the same in the different teeth. In some instances it was pronounced in 2 or 3 teeth but slight in others; or, in some cases there was slight erosion in several of the teeth and none in the remainder. Averaging the degree of erosion of a number of teeth with such variations would ac-

cordingly give fractional values. The fractions, however, are omitted in the table because the nearest whole number is sufficient to indicate whether the extent of erosion was the same or different in the teeth of the rats on the various juices.

The juices are arranged serially in the table in accordance with the increasing loss in weight of the enamel of the lower teeth of the test animals. The loss in weight of the entire tooth and of the enamel was determined by taking the control values as the norms. In the case of the entire tooth it may not have been due in every instance entirely to the loss of the enamel for in some teeth in which the degree of microscopic erosion averaged 3 to 5, the erosion was so deep in places that it seemed to the observer that there had been some destruction of the dentine also.

The erosion produced by tomato and prune juice, as seen under the microscope, was relatively slight whereas with the other juices it was definitely pronounced. As will be observed from a comparison of the data in the table there was good correspondence between the loss in weight of the enamel and the subjective evaluation of the degree of erosion, except in 1 instance. The microscopic erosion produced by pineapple juice was less than that caused by orange juice, whereas the loss in weight of the enamel was slightly greater on the pineapple juice.

The pH of each bottle or can of the juices used in the experiment was determined with the glass electrode. The variation from sample to sample did not exceed 0.3 pH; in most instances, it was within 0.1 pH. Titratable acidity was determined electrometrically by titrating to the pH end-point of the predominating acid.

There seemed to be a general tendency for the degree of erosion to increase with an increase in the acidity of the juice ingested as expressed in terms of either hydrogen ion concentration or per cent titratable acidity. However, it should be noted that there was not a strict and invariable correspondence between the acidity and the amount of tooth destruction. Apple juice, for example, was less acidic than pineapple juice

but produced a much greater amount of erosion and loss of enamel. There was considerable difference in weight loss of enamel caused by prune and orange juice, although the pH of the 2 juices was the same. Similar results were obtained with grape and grapefruit juice.

Part 2. Fruit juices constituting a portion of the total fluid intake

From the standpoint of dietetics, the criticism may be raised against the preceding experiments that in the human consumption of the fruit juices, it is customary to drink them once or at most several times a day. Consideration of this fact suggested the advisability of the following study.

The procedure was the same as in Part 1 except that the test animals were given tomato, orange or grapefruit juice 3 times a day and when this had been consumed were then allowed to drink water *ad libitum*. As in the preceding experiments sugar was added to the grapefruit juice to make it palatable.

Each day except Sundays the test animals were given the juices in an amount equal to one-third the water intake of the preceding day. This was divided into 3 equal portions which were presented to the animals in graduated drinking tubes early in the morning, at noon, and again in the late afternoon. Usually the juices were consumed promptly. This procedure was continued for 200 days, at the end of which time the animals were sacrificed and the teeth removed for study as in the preceding experiment. In the course of the experiment each test animal consumed approximately 1.2 liters of fruit juice which was equivalent to 23% of its total water intake. The total intake of the juices was less than one-third of that of water because water alone was drunk on Sundays.

A comparison of the results presented in table 2 with those in table 1 shows that there was a loss in the weight of the enamel of the test animals on the various juices, but that it was considerably less than when these juices served as the

sole source of fluid. The loss in weight of the entire tooth was extremely small and was not of statistical significance. This was doubtless because the enamel constitutes such a small portion of the entire tooth. A 10% destruction of the enamel would amount to only about 2% of the weight of the entire tooth.

TABLE 2

Effects of various juices on the lower molar teeth of the white rat when ingested 3 times a day for 200 days.

FLUID CONSUMED	WEIGHT OF LOWER MOLARS	LOSS IN WEIGHT	DEGREE OF EROSION	WEIGHT OF ENAMEL	LOSS IN WEIGHT OF ENAMEL	NUMBER OF ANIMALS
	mg	%		*mg*	%	
Water	69.0	..	0	12.6	..	12
Tomato juice	68.2	1.2	0	11.8	6.3	12
Orange juice	67.0	2.9	1	11.0	12.7	12
Grapefruit juice (sweetened)	67.4	2.3	2	10.1	20.0	12

DISCUSSION

The lack of a strict correspondence between the extent of erosion and the degree of acidity of the ingested fluid observed in these experiments would appear to confirm the conclusions of McClure and Ruzicka ('46) that there are other factors besides acidity which produce a decalcification of enamel and dentine *in vivo*. In their experiments the citrate ion had a marked local decalcifying action, whereas no such action was caused by the lactate ion, although the pH of the citrate and lactate drinking fluid was the same. In our experiments there were striking exceptions to what appeared to be a general tendency toward greater decalcification with increasing acidity. Other investigations (Gortner, McCay, Restarski and Schlack, '46) have shown that oxalic acid with a pH as low as 2.1 had no etching action on the enamel of the teeth of rats, but produced a hard deposit which afforded protection against decalcification by phosphoric and citric acid.

Further investigations of the decalcifying action of different ions, as suggested by the work of McClure and Ruzicka

('46) should yield profitable results. Since our experiments were not undertaken with this purpose in mind, there was not sufficient control of certain variables to permit definite conclusions with regard to the action of specific ions. However, it is of interest to re-examine our data in the light of the compilation of the acid constituents of fruits (Hartmann and Hillig, '34) given in table 3.

Juices from the 3 fruits containing the highest concentration of citric acid produced marked erosion. Grapefruit juice, which contains more citric than orange and pineapple juice, caused much greater erosion. However, grape juice which contains practically no citric acid and relatively little malic,

TABLE 3

Acid constituents of various fruit juices (Hartmann and Hillig, '34).

	CITRIC	MALIC	TARTARIC
	%	%	%
Orange, Florida	0.92	0.18	...
Grapefruit	1.33	0.08	...
Grape, Concord	0.02	0.31	1.07
Tomato	0.47	0.05	...
Prune (Italian style)	none	1.44	...
Apple	0.0–0.03	0.27–1.03	...
Pineapple	0.77	0.12	...

but a large amount of tartaric acid caused erosion which was almost as prominent as that produced by grapefruit juice. The results obtained with prune and apple juice are difficult to explain. Neither contains citric acid. Prune juice is much richer in malic acid and yet it produced considerably less erosion than apple juice. Apparently we must look for some other factor than malic acid alone to account for the erosive action of apple juice.

In view of the fact that the erosion of teeth by acid-containing beverages is a topical and not a systemic effect, it was not considered necessary to record the amount of food consumed which, as stated previously, was eaten *ad libitum*. In this connection it should be noted (table 1) that the loss in

weight of the teeth paralleled the degree of erosion as determined by microscopic examination.

It is possible that the sugar in the grapefruit juice may have accentuated the erosive action of the acid in the juice (Gortner, Restarski and McCay, '45). The primary purpose of this investigation, however, was not to study the mechanism of erosion by acid-containing beverages, but to obtain comparative data on the erosive action of various juices which appear to have become fairly common articles of human consumption.

While these and other experiments of a similar nature that have been reported in the literature may ultimately contribute toward the solution of the enigmatic problem of erosion and caries in human teeth, in our opinion, it would be inadvisable to apply them as a guide for a dietary regimen without further extensive investigation. In the first place, the amount of fruit juices ingested by the animals, even in the experiments in Part 2, is relatively much greater than what may be assumed to be the average human consumption. Secondly, the drinking mechanism in the rat is different from that in man. The rat laps its fluid and it is not improbable that the fluid in passing through the mouth may be in contact with the teeth longer than in the human mouth. This is a matter which merits closer study.

SUMMARY AND CONCLUSIONS

Erosion in the lower molar teeth of the albino rat resulted from the daily ingestion of each of the following canned fruit juices when they served as the sole fluid intake over a period of 100 days: apple, grape, orange, tomato, sweetened grapefruit, pineapple and prune.

The greatest amount of erosion occurred in the teeth of the animals on apple, grape, and grapefruit juice, the least in those of the animals on tomato and prune juice.

Erosion of a milder degree also resulted from the ingestion of tomato, orange, and sweetened grapefruit juice 3 times a day for 200 days.

There appeared to be some relationship but not a strict correspondence between the degree of acidity of the juices and the amount of tooth destruction.

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THE PHYSIOLOGICAL AVAILABILITY OF PANTOTHENYL ALCOHOL[1, 2]

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ONE FIGURE

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Relatively few compounds related to pantothenic acid have been found to display vitamin activity. Nease ('43) has listed 26 derivatives and analogues of pantothenic acid which had been tested for pantothenic acid activity by microbiological methods. Only one, hydroxypantothenic acid, showed any significant activity, which varied from 1.5 to 25% of pantothenic acid, depending upon the organism used and the assay conditions (Mitchell et al., '40). Zschiesche and Mitchell ('40) demonstrated that hydroxypantothenic acid has growth-promoting properties for rats also, although of a lesser magnitude than pantothenic acid, which could not, however, be stated quantitatively because of the small number of rats used.

The most active analogue which has been described is pantothenyl alcohol: α,γ-dihydroxy-N-(3-hydroxypropyl)-β,β-dimethylbutyramide, the alcohol analogue of pantothenic acid. Pfaltz ('43) found that pantothenyl alcohol is approximately as effective as calcium pantothenate with regard to influence on growth and achromotrichia of black rats and alopecia in mice. Burlet ('44) demonstrated that pantothenyl alcohol is

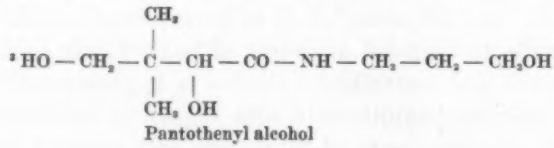
[1] A preliminary report of this work was presented before the Division of Agricultural and Food Chemistry at the 111th meeting of the American Chemical Society, April 14, 1947, Atlantic City, New Jersey.

[2] Roche Publication no. 97.

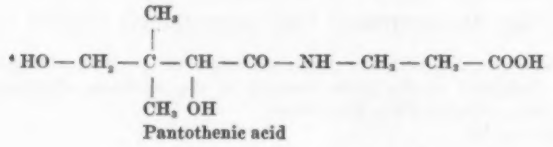
converted by the rat and by man to pantothenic acid, as judged by urinary excretion of pantothenic acid. In another vein, Shive and Snell ('45) reported that pantothenyl alcohol is an inhibitor for several microorganisms at molar inhibition ratios of 700–200,000. The present study concerns further comparative investigation of the physiological availability of pantothenyl alcohol [3] and calcium pantothenate [4] to normal humans.

EXPERIMENTAL

The human availability technique used in these studies was an adaptation of the human bioassay method of Melnick et al. ('45). Seven healthy male adult subjects participated in these excretion experiments. Pantothenic acid in the urines was determined by the method of Atkin et al. ('44) employing *Saccharomyces carlsbergensis* 4228, and by the method of Skeggs and Wright ('44) employing *Lactobacillus arabinosus* 17-5. Pantothenyl alcohol or pantoyl lactone at levels of 0.05 to 0.5 μg per tube, with or without equal amounts of pantothenic acid, did not stimulate or inhibit either of these 2 organisms. Extensive assays of basal urines of all subjects prior to actual dosage yielded an average value of 3.7 mg per day (range 2.1–5.0). These values are of the same order as those reported by previous workers (Sarett, '45). The diets were not controlled rigorously although each subject avoided foods high in pantothenic acid and vitamin supplements. Twenty-four hour samples of urine were collected in amber jars containing 10 ml of toluene, the first voiding of the first



Pantothenyl alcohol



Pantothenic acid

day being discarded. Equivalent doses of d(+)-pantothenyl alcohol or calcium d(+)-pantothenate, dissolved in distilled water, were taken orally immediately after the midday meal and the urine collections continued for either 1, 3 or 5 consecutive days. The dose-response relationship was established by feeding various levels of calcium pantothenate and measuring the extra urinary pantothenic acid excretion; this relationship is illustrated graphically in figure 1.

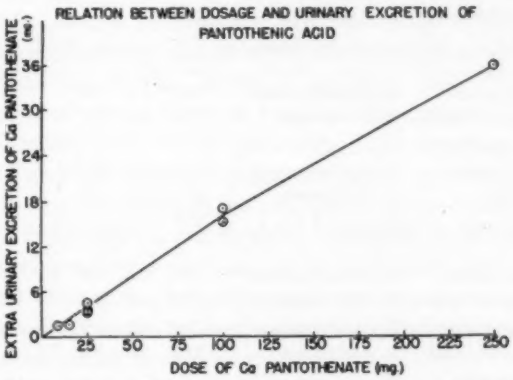


Fig. 1 Relation between dosage and urinary excretion of pantothenic acid.

Burlet ('44) demonstrated a greater excretion of pantothenic acid (ca. 50–100%) in the urines of 2 young men given 250 mg of pantothenyl alcohol as compared with 250 mg of calcium pantothenate. In this laboratory, 100 mg of calcium pantothenate (equivalent to 0.42 mM of pantothenic acid) was chosen as the first test dose and the urines were collected for a 24-hour period. This dose provides an excretion which is on the linear portion of the dose-response curve and which is large enough to minimize any errors due to possible fluctuations in the basal excretions. One week later, an equivalent amount of pantothenyl alcohol (86 mg) was given to the same subjects and the urines were collected for 24 hours. A summary of the data is presented in table 1, from which it is apparent (experiment I) that a decidedly greater excretion

TABLE 1

Summary of human availability studies at dosage levels of 0.42 mM and 1.05 mM taken after lunch.

EXPT. NO.	SUBSTANCE TAKEN	ASSAY ORGANISM	EXTRA EXCRETION OF PANTOTHENIC ACID IN URINE IN MG/DAY ON DAY					TOTAL EXTRA EXCRETION IN MG
			1	2	3	4	5	
I	100 mg calcium pantothenate	S. Carlsbergensis	11.5					11.5
		L. Arabinosus	11.3					11.3
	86 mg pantothenyl alcohol	S. Carlsbergensis	18.8					18.8
		L. Arabinosus	21.3					21.3
	Availability of pantothenyl alcohol $= \frac{20.1}{11.4} \times 100 = 176 \pm 28\%$ [1]							
II	86 mg pantothenyl alcohol	S. Carlsbergensis	19.3	3.9	3.3			26.5
		L. Arabinosus	21.7	4.4	3.0			29.1
	100 mg calcium pantothenate	S. Carlsbergensis	17.8	5.1	2.5			25.4
		L. Arabinosus	19.2	5.0	2.3			26.5
	Availability of pantothenyl alcohol $= \frac{27.8}{26.0} \times 100 = 107 \pm 6.9\%$ [1]							
III	86 mg pantothenyl alcohol	S. Carlsbergensis	18.9	2.3	2.0	1.0	1.0	25.2
		L. Arabinosus	18.7	1.6	1.2	0.4	0.4	22.3
	86 mg pantothenyl alcohol	S. Carlsbergensis	19.5	3.2	1.9	0.7	0.6	25.9
		L. Arabinosus	18.5	2.7	2.2	1.8	1.4	26.6
	100 mg calcium pantothenate	S. Carlsbergensis	17.0	2.3	0.9	0.4	0.8	21.4
		L. Arabinosus	16.9	2.1	0.4	0.4	1.4	21.2
	Availability of pantothenyl alcohol $= \frac{25.0}{21.3} \times 100 = 117 \pm 8.5\%$ [1]							
IV	250 mg calcium pantothenate	S. Carlsbergensis	38.8					38.8
		L. Arabinosus	44.3					44.3
	215 mg pantothenyl alcohol	S. Carlsbergensis	61.1					61.1
		L. Arabinosus	66.4					66.4
	215 mg pantothenyl alcohol	S. Carlsbergensis	61.4					61.4
		L. Arabinosus	68.0					68.0
	250 mg calcium pantothenate	S. Carlsbergensis	32.4					32.4
		L. Arabinosus	34.5					34.5
	253 mg sodium pantothenate	S. Carlsbergensis	33.7					33.7
		L. Arabinosus	39.9					39.9
	Availability of pantothenyl alcohol $= \frac{64.2}{37.5} \times 100 = 171 \pm 14.7\%$ [1]							
	Availability of sodium pantothenate $= \frac{36.8}{37.5} \times 100 = 98 \pm 13.3\%$ [1]							

[1] Standard error of the quotient.

of pantothenic acid occurred after ingestion of pantothenyl alcohol than after the ingestion of an equivalent amount of calcium pantothenate. These results apparently were in good agreement with those of Burlet ('44).

In order to test whether the greater excretion of pantothenic acid after the ingestion of pantothenyl alcohol might be due to more rapid excretion, the experiment was repeated with the urine collection period extended to 3 consecutive days. The results are given in table 1 (experiment II). In this experiment, contrary to the first one, the large differences between the pantothenic acid excretions disappeared after the ingestion of equivalent doses of calcium pantothenate and pantothenyl alcohol. This is true of the first-day excretion as well as of the total 3-day excretion. It is therefore probable that a differential rate phenomenon is not involved, but that the degree of saturation of the subjects is important. The first test dose (calcium pantothenate) shows a markedly lower level of excretion than the second, third and fourth test doses (pantothenyl alcohol and calcium pantothenate), and since the latter are comparable to each other, a priming dose of about 100 mg is indicated.

With this in mind, the experiment was repeated with urine collections continued for 5 consecutive days. Two trials with pantothenyl alcohol were made 1 week apart and then 1 trial with calcium pantothenate. Determinations of basal values during and after this experiment revealed a slight increase in basal pantothenic acid excretion; these higher figures were used in computing the extra urinary excretion. Since the 2 trials with pantothenyl alcohol agreed very well, the results were averaged and then compared with the excretion after the dose of calcium pantothenate was given. The results are shown in table 1, wherein it can be seen that the results of the second and third experiments are in good agreement and indicate a slight superiority of pantothenyl alcohol over calcium pantothenate at the level fed.

It is apparent from the results of the second and third experiments in table 1 that the physiological availability of

pantothenyl alcohol lies between 107 and 117% of that of calcium pantothenate under the stated conditions. The first experiment is not included in this calculation, since, as stated above, the subjects were apparently not saturated with respect to calcium pantothenate.

Since Burlet ('44) had found a greater excretion of pantothenic acid after ingestion of a 250 mg dose of pantothenyl alcohol than after an equal weight of calcium pantothenate was given, the excretion experiments were extended with doses of 250 mg of calcium pantothenate and equivalent amounts of pantothenyl alcohol (215 mg) taken alternately 1 week apart. Basal urines were collected on the first day, the dose given on the next day, and the urines again collected during the next 24 hours. The results (table 1, experiment IV) show, in agreement with Burlet, that considerably more pantothenic acid is excreted after dosages of pantothenyl alcohol at this level.

The role of the cation was studied in an experiment with an equivalent dose of sodium pantothenate. From table 1 it is evident that the relative availability of sodium pantothenate is of the same order as that of calcium pantothenate, and the relative availabilities of both are distinctly lower than that of pantothenyl alcohol at this dose of 1.05 mM. Therefore the presence of calcium in the molecule is probably not a factor in the lower excretion as compared to pantothenyl alcohol when the test doses are taken after lunch, as was the case in the preceding experiments.

Silber ('45) has demonstrated that the presence of food in the gastrointestinal tract diminishes the absorption of calcium pantothenate in dogs fed amounts comparable to those in this experiment, namely 4 mg per kilo. As the doses in the present experiments were given directly after the midday meal, it was thought that this factor might be operative. Trials were therefore undertaken in which the doses were given on an empty stomach. The subjects refrained from eating breakfast on the morning of the experiment, took either 215 mg of pantothenyl alcohol or 250 mg of calcium pantothenate at 9 A.M., and

continued fasting until 12:30 P.M. Urines were collected for 24 hours. Complete excretion data for the first experiment and a summary of data for a second experiment are shown in table 2. The average urinary excretion of pantothenic acid by all subjects is greater after ingestion of the pantothenyl alcohol than after calcium pantothenate was given under these conditions. However, the excretions of 2 subjects, E.D.R. and S.H.R., are distinctly lower than those of the others after ingestion of calcium pantothenate but are within the general range after pantothenyl alcohol was administered. If the excretion values are averaged without these subjects, the differences between the excretions largely disappear after doses of the 2 compounds. The experiment was repeated with the dose of calcium pantothenate given first and again these 2 subjects excreted lesser amounts of pantothenic acid after taking calcium pantothenate. A summary of the relative availability values for pantothenyl alcohol is given in table 2. The average availability in experiments I and II, including all subjects, is $130 \pm 31\%$. However, when the values for the excretion of the 2 subjects, E.D.R. and S.H.R., after doses of both pantothenyl alcohol and calcium pantothenate are excluded, the availability of pantothenyl alcohol is $101 \pm 12\%$. Apparently these 2 subjects utilized pantothenic acid less efficiently than pantothenyl alcohol when ingested in the postabsorptive state.

Further inspection of table 2 shows that the values for extra excretion of pantothenic acid obtained by assay with *L. arabinosus* are higher than those obtained with *S. carlsbergensis*. A statistical analysis of these differences is given in table 3. After the lesser dose of calcium pantothenate, the difference is not significant, but at the lower level of pantothenyl alcohol the difference is significant at the 5% level but not at the 1% level. At the higher levels of both calcium pantothenate and pantothenyl alcohol, the differences are highly significant even at the 1% level.

Since these differences were greater after the dosage with pantothenyl alcohol, it was thought that perhaps some panto-

TABLE 2

One-day excretion of pantothenic acid after a dose of 215 mg pantothenyl alcohol or 250 mg calcium pantothenate taken in the postabsorptive state. All values are in milligrams.

SUBJECT	DOSE: 215 MG PANTOTHENYL ALCOHOL						DOSE: 250 MG CALCIUM PANTOTHENATE					
	Assay with S. Carlsbergensis			Assay with L. Arabinosus			Assay with S. Carlsbergensis			Assay with L. Arabinosus		
	Excretion:			Excretion:			Excretion:			Excretion:		
	Basal	Total	Extra	Basal	Total	Extra	Basal	Total	Extra	Basal	Total	Extra
Experiment I												
E.D.R.	5.9	71.8	65.9	6.9	108.0	101.1	8.4	17.5	9.1	10.4	19.9	9.5
J.M.C.	9.1	69.5	60.4	11.8	86.3	74.5	10.7	71.5	60.8	13.3	76.7	63.4
E.H.	5.5	62.3	56.8	5.8	67.2	61.4	7.9	73.3	65.4	8.5	75.8	67.3
S.H.R.	3.1	80.6	77.5	4.4	89.9	85.5	4.6	47.9	43.3	5.3	46.2	40.9
J.S.	6.5	81.4	74.9	7.7	93.8	86.1	9.2	91.7	82.5	9.7	93.9	84.2
F.W.J.	6.5	94.5	88.0	7.3	120.0	112.7	9.1	83.5	74.4	11.2	88.9	77.7
Average	6.1	76.7	70.6	7.3	94.2	86.9	8.3	64.2	55.9	9.7	66.9	57.2
Average excluding E.D.R. and S.H.R.	6.9	76.9	70.0	8.1	91.8	83.7	9.2	80.0	70.8	10.7	83.8	73.1
Experiment II												
Average	8.0	74.1	66.1	9.1	82.7	73.6	7.0	65.2	58.2	7.2	64.5	57.3
Average excluding E.D.R. and S.H.R.	9.0	81.8	72.8	9.2	91.4	83.2	9.1	93.4	84.3	9.2	89.6	80.4

Per cent relative availability of pantothenyl alcohol [1]

	Organism	Experiment I	Experiment II	Mean
All subjects	S. Carlsbergensis	126 ± 26	114 ± 33	120 ± 30
	L. Arabinosus	152 ± 33	128 ± 33	140 ± 33
	Mean	139 ± 30	121 ± 33	130 ± 31
E.D.R. and S.H.R. excluded	S. Carlsbergensis	99 ± 12	88 ± 11	94 ± 12
	L. Arabinosus	114 ± 13	102 ± 11	108 ± 12
	Mean	107 ± 13	95 ± 11	101 ± 12

[1] $\frac{\text{Excretion after pantothenyl alcohol}}{\text{Excretion after Ca pantothenate}} \times 100 \pm$ standard error of the quotient.

thenyl alcohol as such or pantoyl lactone was being excreted into the urine and might be acting as a stimulator for the organisms. Accordingly, to 3 of the urines which showed the greatest variation in level of pantothenic acid by the 2 organisms, were added equivalent amounts of pantothenyl alcohol, and to a duplicate series one-half an equivalent amount of pantothenyl alcohol and one-half an equivalent

TABLE 3

Estimates of significance of differences between urinary pantothenate assays by L. Arabinosus and S. Carlsbergensis.

SUBSTANCE TAKEN	DOSE	NO. SAMPLES	AVERAGE PANTOTHENATE ASSAY			SIGNIFICANCE OF DIFFERENCE (t)	VALUE OF t FOR SIGNIFICANCE [1]	
			L. Arabinosus	S. Carlsbergensis	$\frac{\text{L. Arabin.}}{\text{S. Carlsb.}} \times 100$		5% level	1% level
	mg		*mg*	*mg*	%			
Calcium pantothenate	100	21	21.9	21.5	102	0.34	2.086	2.845
Calcium pantothenate	250	17	63.7	59.8	107	6.89	2.120	2.921
Pantothenyl alcohol	86	25	24.8	23.7	105	2.44	2.064	2.797
Pantothenyl alcohol	215	24	82.0	72.5	113	6.28	2.069	2.807

[1] Snedecor, G. W., Statistical Methods, p. 58, Collegiate Press, Inc., Ames, Iowa, 1938.

amount of pantoyl lactone. The urines were then diluted and assayed both by *L. arabinosus* and *S. carlsbergensis*. In no case, however, was there any significant difference from the values obtained in the assay of the original urines.

Wood ('47) has described a method for the computation of microbiological assays which also tests the validity of the assay. In this method the response is plotted against the logarithm of the dose. The pantothenic acid content of the

3 urines mentioned above was computed by this method as well as by the single curve method with essentially similar results.

DISCUSSION

The results show that with increasing dosage of the 2 compounds, pantothenyl alcohol shows increasingly superior availability when taken together with food. The connotations attached to the term "availability" in these experiments are somewhat different than usual, in that we are not concerned with the utilization of the same compound in different media, but rather in the behavior of 2 related compounds in the same medium.

Several factors may be considered in connection with the greater availability of pantothenyl alcohol. The evidence from the experiments in the postabsorptive state (table 2) and from Silber's ('45) experiments with dogs favors the view that, in the presence of food, calcium or sodium pantothenate is lost in the feces more readily than pantothenyl alcohol. Another possibility arises from the better stability of pantothenyl alcohol in acid solutions. It is well-known that pantothenic acid is readily hydrolyzed in dilute acid (Atkin et al., '44); experiments in this laboratory have shown that pantothenyl alcohol is considerably more stable than pantothenic acid in dilute acid, ca. pH 2 to 5 (unpublished data). It is therefore possible that greater destruction of the latter occurs in the stomach when the test doses are taken directly after lunch (table 1) than when taken in the postabsorptive state (table 2).

SUMMARY

The urinary excretion of pantothenic acid by 7 normal male subjects was slightly less after an oral dose of 100 mg calcium pantothenate than after an equivalent dose of pantothenyl alcohol, when taken after lunch. A priming dose of calcium pantothenate is necessary to insure valid results.

When the dose was increased to 250 mg of calcium pantothenate or 215 mg of pantothenyl alcohol, a decidedly greater excretion of pantothenic acid occurred after the ingestion of

pantothenyl alcohol. Similar results were obtained when sodium pantothenate was substituted for calcium pantothenate.

When the subjects received the greater dosages in the post-absorptive state, the differences in the pantothenic acid excretion disappeared largely except in the case of 2 of the 7 subjects.

The *L. arabinosus* assay values for pantothenic acid in the urines following the ingestion of the greater doses of both calcium pantothenate and pantothenyl alcohol were significantly higher than those obtained with *S. carlsbergensis*.

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THE ENERGY EXPENDITURE FOR QUIET PLAY AND CYCLING OF BOYS SEVEN TO FOURTEEN YEARS OF AGE [1]

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ONE FIGURE

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Since the time of Lavoisier the energy requirement for different activities has been actively investigated but by far the greater part of the work has been carried out on adult subjects. The energy cost of many children's activities has not as yet been measured. This paper reports the results of measuring the energy expenditure of 7- to 14-year-old boys (a) sitting at quiet play and (b) cycling in the respiration chamber of the Nutrition Laboratory of Teachers College, Columbia University. Those reports found in the literature with which these results could be compared are cited under Results and Discussion.

EXPERIMENTAL

Fifteen boys living in 2 homes for children in New York City served as subjects. Six of these boys served in successive years and were considered as new subjects each year, thus bringing the total number of cases up to 22. They were selected

[1] The data in this paper were taken from dissertations submitted by Clara M. Taylor ('37), Mina W. Lamb ('42) and Mary E. Robertson ('42) in partial fulfillment of the requirements for the degree of Doctor of Philosophy, under the Joint Committee on Graduate Instruction, Columbia University.

on the basis of age, height, weight, good health, and general spirit of cooperativeness displayed during preliminary tests. All of the boys were born in this country but varied in racial background. Three age groups were studied. Data for each group, with respect to age range, average weight, height, and deviation from predicted weight (Baldwin-Wood standards) are given in table 1.

TABLE 1

Average weight, height, deviation from predicted weight, and basal metabolism of boys. (Figures in parentheses show range.)

SUBJECTS	BODY WEIGHT		BODY HEIGHT		DEVIATION FROM PREDICTED WEIGHT [1]	BASAL METABOLISM IN CALORIES PER			
						24 hr.	kg/hr.	sq.m./hr.	cm of ht./24 hr.
	lb	*kg*	*inch*	*cm*	%				
Group I: 6 subjects with ages ranging 6–6 to 8–4 [2]	47.6 (41.1–56.1)	21.6 (18.7–25.5)	46.4 (42.5–50.4)	117.9 (108.1–127.9)	—2.9 (—5.2–+2.8)	1030 (881–1087)	2.0 (1.7–2.2)	51.7 (43.9–54.5)	8.8 (7.9–9.2)
Group II: 6 subjects with ages ranging 9–5 to 11–1 [2]	63.3 (50.6–72.0)	28.8 (23.0–32.7)	52.0 (49.4–54.7)	132.0 (125.5–138.8)	—1.3 (—10.1–+4.3)	1101 (1009–1309)	1.6 (1.4–1.9)	44.4 (41.2–48.2)	8.3 (7.8–9.5)
Group III: 10 subjects with ages ranging 12–4 to 14–1 [2]	90.3 (68.1–117.8)	41.0 (31.0–53.6)	58.2 (54.7–64.4)	149.1 (138.9–163.6)	+1.0 (—6.9–+15.3)	1277 (1059–1604)	1.3 (1.2–1.5)	40.6 (38.9–43.9)	8.5 (7.6–9.8)

[1] Deviations from Baldwin-Wood standards.

[2] First number in each set refers to years, the second to months.

The basal metabolism was determined by means of the Sanborn-Benedict portable respiration apparatus, the Collins-Benedict-Roth respiration apparatus, or the Benedict and Benedict student respiration apparatus. Tests with the same

subject on these 3 forms of apparatus in this laboratory have repeatedly demonstrated that they may be used interchangeably for basal energy metabolism determinations. The determinations were made under the generally accepted standard conditions, i.e., in the period 12 to 18 hours after the last intake of food, lying still awake after a 30-minute rest period, body temperature normal and environmental temperature comfortable. Two 8- to 10-minute determinations were made each morning and the procedure repeated on other mornings until at least 3 of the lowest results were found to check within 5%. In most cases 2 mornings were sufficient to obtain acceptable results. The average results expressed in 4 ways are given in table 1. These averages have been used for comparison with the energy expenditure for quiet play and cycling.

The activity studies were carried out in an open circuit respiration chamber patterned after the respiration chambers used by Benedict et al. ('20) and Ritzman and Benedict ('29). The original chamber was described in Columbia University dissertations by Potgieter ('33), Robb ('34), and Williams ('34). After the completion of their studies, the chamber was moved and some alterations made in dimensions and lay-out. These new dimensions and lay-out were given by Taylor ('37). A diagram of the chamber with bicycle ergometer in place, the absorbing trains for carbon dioxide and the remote control board is shown in figure 1. The dimensions of the chamber are as follows: length, 222.3 cm; width, 171.0 cm; height, 226.8 cm; volume, 8,577 l.

At frequent intervals (usually once a week) the absorbing trains and the respiration chamber were tested for tightness by introducing a weighed amount of carbon dioxide directly into the chamber through the opening at "h" (fig. 1). A sample of the air in the chamber was taken before the carbon dioxide was introduced and again at the end of a 25-minute period of circulation through the absorbers to determine the percentage of carbon dioxide in the residual air of the chamber. The increase in grams of carbon dioxide in the

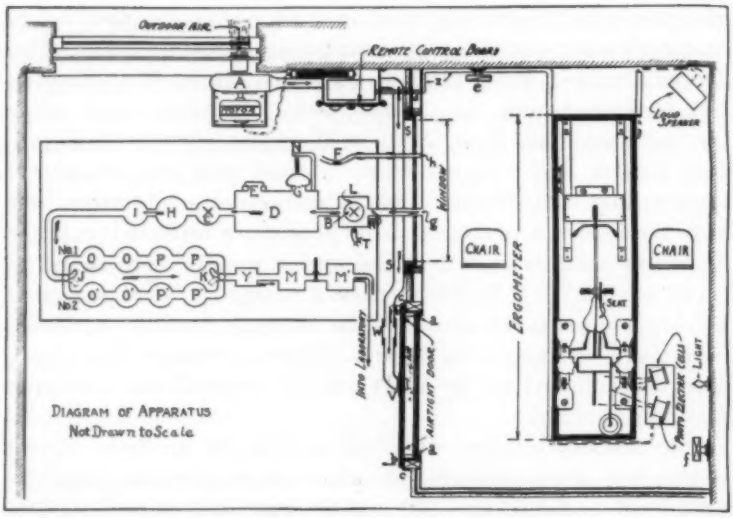

Fig. 1 Diagram of Apparatus. A, blower for drawing in outdoor air and forcing it into chamber; B, blower for removing air from chamber and carrying it to wind chest; C, positive blower for forcing air from wind chest D through absorbing systems; E, metal cap closing wind chest; F, manometer for testing pressure of chamber air; G, rubber cap attached to spring for maintaining air pressure in chamber; H, large jar of sulfuric acid for drying chamber air; I, small jar of sulfuric acid, an added precaution for drying air; J, K, levers directing chamber air through absorbing train, I or II; L, tin box acting as housing for blower B; M, M^1, meters for measuring volume of air passing through chamber; N, support for spring and rubber cap; O, O and O^1, O^1, jars of soda lime for absorbing CO_2; P, P and P^1, P^1, jars of H_2SO_4, for absorbing moisture; R, metal tube connecting chamber with blower B; S, metal tubing for conducting outdoor air to chamber; T, small opening for introducing CO_2 for testing absorbing train; V, opening into door, permitting entrance of outdoor air; W, rubber tubing completing air passage into chamber; Y, metal receptacle containing cotton sprinkled with $NaHCO_3$ for neutralizing acid fumes in air leaving sulfuric acid jars; Z, electric cable carrying electric connections for magnetic core, photo-electric cell, red and green lights and loud speaker; a, a and b, b, felt connections on door frame; c, c, clamps for holding chamber door in place; d, opening in chamber wall for passage of electric cable; e, f, electric fans for evenly distributing chamber air; g, opening through which chamber air is drawn into absorbing system; h, opening for taking samples of chamber air.

chamber air plus the corrected grams of carbon dioxide absorbed by the absorbing system gives the total return on the weighed amount of carbon dioxide introduced. The average recovery of carbon dioxide throughout the investigations ranged between 99.3 and 99.9%.

The Carpenter ('23, '29, '33) modification of the Haldane gas analysis apparatus was used. This apparatus measures the carbon dioxide in air to the nearest 0.001%. Only those results agreeing within 0.004% were considered acceptable. The apparatus was tested for accuracy at frequent intervals by analyzing samples of outdoor air. Carpenter suggests that if a given sample of outdoor air is found to contain between 0.027 and 0.033% of carbon dioxide the apparatus may be considered to be in good working condition.

Plunger pumps, described by Lee ('33), were used for sampling the chamber air. The pumps were tested for tightness before each experiment. Usually the gas samples were analyzed within a few hours, although it was established that samples allowed to stand for as long as 24 hours showed no change in composition. These pumps have a capacity of 370 ml so that it is possible, if necessary, to make as many as 5 determinations on 1 sample.

For the cycling tests a bicycle ergometer [2] was used which was patterned after the bicycle ergometers of electric brake type designed by Benedict and Carpenter ('09), Benedict and Cady ('12) and Krogh ('13). Detailed description is given by Taylor ('37). In order that approximately the same amount of work should be done in each test period, a fairly constant speed of pedaling was maintained by placing a red and a green light on a support just below the handle bars where they could be easily seen by the cyclists. If the pedaling was too fast, the red light would show, and if too slow, the green light. The boys were instructed to pedal just fast enough to keep both lights out. This maintained a speed of 54 to 65 pedal revolu-

[2] The bicycle ergometer was built by personnel furnished by the Works Division of the Emergency Relief Bureau of New York City under the direction of Dr. Irving Lorge, Professor of Education, Teachers College, Columbia University.

tions per minute. This device interested the boys and resulted in steadier pedaling than when a metronome was used. The pedaling was done against the electric brake by passing a constant current of 0.6 ampere through the coils of the magnetic core between the pole faces of which the copper disc, which replaced the rear wheel of the bicycle, rotated (fig. 1). This was judged by experienced cyclists to simulate closely the work done against friction of tires and resistance of wind under ordinary conditions of cycling in open country.

In order to have some means of direct communication with the subjects in the chamber, a loud speaker was placed in 1 corner (fig. 1), and connected with a hand microphone which was hung on the control board.

PROCEDURE

The results reported in this paper are those of tests made in the afternoon after school, 3½ to 4 hours after a light to moderate lunch. At the beginning of the study it was established by Taylor ('37), by making tests on the same subjects, 17 in the morning before breakfast and 29 after school, that the agreement of results was so close as to justify the assumption of an average respiratory quotient of 0.82 in the afternoon after school. It was also established, as the result of 15 tests on each of the 3 age groups studied, that cycling at the rate of 49 to 66 pedal revolutions per minute did not cause oxygen debt. Hence, no recovery period was required and it was allowable to use the increase in carbon dioxide production during the cycling periods without making any correction for development of an oxygen debt or irregular production of carbon dioxide.

Two boys were studied at a time and treated as a single subject. Preceding each test, the boys were weighed and their heights determined. They were then given something to provide amusement for the quiet play period, the nature of this being limited to games, puzzles and other quiet activities, which would involve, for the most part, the use of only the

finger muscles with occasional arm movements. Also, they were told to keep their hands on the handle bars while cycling.

The chamber was thoroughly ventilated before each test and the ventilation continued for a 5-minute adjustment period after the boys had entered the chamber and the door had been closed. Then followed the quiet play period, lasting 15 minutes, at the end of which the signal to start pedaling was given. In groups II and III each boy pedaled for 15 minutes, making a total of 30 minutes of cycling. While 1 boy cycled, the other continued sitting at quiet play. In group I, it was found that a 15-minute cycling period was too long and consequently the 30-minute cycling period was divided so that each child in turn rode for 8 minutes and then for 7 minutes, making a total of 15 minutes each. During the 50 minutes when the boys were in the chamber, they were, on the whole, most cooperative.

Samples of chamber air were withdrawn by pumps at the beginning and end of each period and analyzed to obtain the increase in carbon dioxide content of the chamber air. These results were then corrected for the amount of carbon dioxide in the outdoor air circulated through the chamber during the tests. The absorbing trains (fig. 1) were weighed before the beginning and at the end of each period, one being used for the period of quiet play and the other for cycling. To obtain the total carbon dioxide produced, the increase in weight of each absorbing train, corrected for the amount of carbon dioxide of the outdoor air, was added to the corrected carbon dioxide of the chamber air. To obtain the average carbon dioxide produced per boy during the period of quiet play, the total carbon dioxide produced during the period was divided by 2. To obtain the average amount of carbon dioxide produced per boy cycling, the average carbon dioxide produced per boy sitting at quiet play for 30 minutes was deducted from the total carbon dioxide produced during the 30-minute cycling period.

RESULTS AND DISCUSSION

The average results of 70 determinations of energy expenditure for quiet play and of 61 for cycling, including the cost of the basal metabolism, are given in table 2 along with the probable error of the mean, the coefficient of variation, and the probable error as percentage of the mean.

TABLE 2

Average energy expenditure of boys for quiet play and cycling. (Including the basal metabolism.)

	QUIET PLAY			CYCLING		
	Group I (7-8 yrs.)	Group II (9-11 yrs.)	Group III (12-14 yrs.)	Group I (7-8 yrs.)	Group II (9-11 yrs.)	Group III (12-14 yrs.)
CO_2 (gm) produced per child per hr.						
Mean ± P.E.[1]	23.06	25.24	28.63	48.77	50.59	61.37
	±0.41	±0.26	±0.69	±0.75	±0.63	±1.05
No. of cases	19	29	22	19	20	22
C.V.[2] (%)	11.7	8.3	16.8	10.0	8.3	11.9
P.E. as % of mean	1.8	1.0	2.4	1.5	1.2	1.7
Cal. per kg per hr.						
Mean ± P.E.	3.1	2.6	2.1	6.6	5.1	4.5
	±0.07	±0.02	±0.04	±0.13	±0.04	±0.06
No. of cases	19	29	22	19	20	22
C.V. (%)	14.2	6.5	13.3	12.3	5.7	9.3
P.E. as % of mean	2.3	0.8	1.9	2.0	0.8	1.3
Cal. per cm of ht. per hr.						
Mean ± P.E.	0.59	0.58	0.58	1.23	1.15	1.24
	±0.01	±0.01	±0.01	±0.02	±0.01	±0.02
No. of cases	19	29	22	19	20	22
C.V. (%)	11.9	6.9	15.5	10.6	7.0	9.7
P.E. as % of mean	1.7	1.7	1.7	1.6	0.9	1.6

[1] Probable error.

[2] Coefficient of variation.

Considering the variability in the amount of involuntary movement of head, hands, and feet observed from day to day, coefficients of variation ranging from 5.7 to 16.8% are not great. That the coefficients of variation are in general lower

in the cycling period than in the period of quiet play may be due to activity being more closely controlled in the cycling period. The probable error of the mean expressed as per cent of the mean ranges from 0.8 to 2.4%, indicating the validity of the average results.

It will be noted that whereas energy expenditure for each activity in Calories per kilogram per hour decreases with the increase in age, there is practically no change when it is expressed in Calories per centimeter of height per hour.

Comparing the total cost of quiet play with the basal energy expenditure, the percentage increase for the 7- to 8-year-old boys is 55%, for the 9- to 11-year-old boys, 63%, and for the 12- to 14-year-old boys, 62%. For cycling, the increases above the basal metabolism are, respectively, 230, 219 and 246%.

There are few results in the literature with which to compare the findings of this study. For boys in the age range of group I Sondén and Tigerstedt (1895) reported an expenditure of 3.46 Cal. per kg per hr. for sitting quietly reading; Bedale ('23), for sitting quietly in the schoolroom, 2.40; for the quiet play of this study the average result was 3.13. For boys 9 to 11 years of age, sitting reading and eating apples Sondén and Tigerstedt reported 2.75; Olin ('16), for sitting quietly after breakfast, 1.81; Bedale, for sitting quietly in the schoolroom, 2.16; for the quiet play of this study the average result was 2.55. For 14- and 15-year-old boys sitting quietly, Bedale reported 1.96 and 1.90, respectively; for the quiet play of the 12- to 14-year-old group of this study the average result was 2.09. There are no reports in the literature on the energy expenditure of boys in cycling with which to compare the results of this study.

SUMMARY

Seventy determinations of energy expenditure for quiet play and 61 for cycling by 22 subjects, 7 to 14 years of age, are reported. A respiration chamber equipped with a bicycle ergometer of electric brake type was used. The results are

stated as the total expenditure, i.e., inclusive of the basal metabolism.

Nineteen determinations were made on boys 7 and 8 years of age. Sitting quietly at play the average energy expenditure of these boys was 3.1 Cal. per kg per hr., or 0.59 Cal. per cm of ht. per hr. For cycling, the average was 6.6 Cal. per kg per hr., or 1.23 Cal. per cm of ht. per hr.

Twenty-nine determinations of the energy expenditure in quiet play and 20 determinations for cycling were made on boys 9 to 11 years of age. For quiet play the average expenditure was 2.6 Cal. per kg per hr., or 0.58 Cal. per cm of ht. per hr.; for cycling, 5.1 Cal. per kg per hr., or 1.15 Cal. per cm of ht. per hr.

Twenty-two determinations were made on boys 12 to 14 years of age. For quiet play the average was 2.1 Cal. per kg per hr., or 0.58 Cal. per cm of ht. per hr.; for cycling, 4.5 Cal. per kg per hr., or 1.24 Cal. per cm of ht. per hr.

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